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TITLE: **DETECTION AND TREATMENT OF GLYCO-ENZYME-RELATED DISEASE**

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DETECTION AND TREATMENT OF GLYCO-ENZYME-RELATED DISEASE

PRIOR APPLICATIONS

This application is a continuation-in-part of U.S. 08/969,437 filed November 11, 1997 and U.S. provisional application 60/171,728 filed December 22, 1999.

FIELD OF THE INVENTION

The present invention relates to the prevention and treatment of disease by altering glyco-enzyme expression in a cell.

BACKGROUND OF THE INVENTION

Cell surface glycoproteins and glycosphingolipids appear to play an important role in a diverse array of cellular functions including regulation of cell growth, differentiation and intercellular communication (Moskal, 1987; Hakomori, 1981). Glycosylation is known to play various roles in host cell-viral interactions, immune cell recognition and migration, neural cell adhesion and function and the function of gonadotropic hormones (Rademacher, 1988). A defect in the glycosyltransferase function has been associated with several inherited diseases. Congenital dyserythropoietic disease, a condition in which abnormal morphologies are detected in various immune cells is observed, has been attributed to a deficiency of GlcNAc transferase II ("GnTII") (Fukuda, et al. 1987. *J. Biol. Chem.* 262:7195-7206). I-cell disease and pseudo-Hurler polydystrophy, involving a deficiency of phospho-N-acetylglucosaminyl transferase activity, are also genetic diseases involving defective oligosaccharide biosynthesis (Kornfeld, 1986. Clin. Invest. 77:1-6).

Alterations in the expression of terminal sialic acid residues on glycoconjugates are common phenomena in oncogenic transformation (Kaneko, 1996; Nicholson, 1982; Roth, 1993; Schirrmacher, 1982; Varki, 1993). Increased cell-surface sialylation has been implicated in invasivity (Collard, 1987), tumor cell-mediated platelet aggregation (Bastida, 1987), resistance to T-cell mediated cell death (Workmeister, 1983), adhesion to

endothelial cells and extracellular matrices (Dennis, 1982), and metastatic potential (Passanti, 1988). Studies have shown a correlation between increased terminal sialylation of cell-surface glycoproteins and both the metastatic and invasive potential of a variety of tumors (Collard, 1987; Nicholson, 1982; Passanti, 1988, Varki, 1993). It has also been reported that terminal sialylation of glycoproteins found in human chronic myelogenous leukemia K562 cells increases their resistance to T-cell-mediated cell lysis (Workmeister, 1987).

At least ten distinct enzymes are known to transfer sialic acid to the termini of the oligosaccharide moieties of glycosphingolipids and glycoproteins, termed sialyltransferases. These enzymes comprise a structurally related family of molecules that display substrate specificity, tissue specificity, and are developmentally regulated (Kitagawa, 1994). There are at least two sialyltransferases which transfer sialic acid to the nonreducing termini of sugar chains of N-linked glycoproteins. One is CMP-NeuAc:Galβ1,3(4)GlcNAc α2,6-sialyltransferase (α2,6-ST); another is CMP-NeuAc:Galβ1,3(4)GlcNAc α2,3-sialyltransferase (α2,3-ST). These transferases have been shown to be cell-type specific and appear to modulate a variety of important cellular processes. It is currently appreciated by those skilled in the art that alterations in the glycosylation of cell surface molecules involved in invasivity (e.g., gangliosides, growth factor receptors, etc.) may have a distinct effect on the tumorigenic and metastatic potential of tumor cells.

Presently, treatment of neurological disorders such as brain cancer is limited in its efficacy and there is a need in the field for efficient and successful strategies for treating such disorders. While a number of investigators have used cell lines derived from vertebrate brain tumors to study the expression and regulation of various glycosyltransferases (Demetriou, 1995; Takano, 1994; La Marer, 1992), studies using primary human brain tumor material have been very limited. Shen et al. (1984) reported that serum sialyltransferase, using desialylated fetuin as the acceptor, did not significantly differ from controls in glioma patients. Gornati et al. (1985) found that the sialyltransferase involved in the biosynthesis of GD3 from GM3 ganglioside was altered in meningiomas.

The present application provides a methodology that, in at least one embodiment, involves transfer of a gene encoding a protein having sialyl- or glycosyltransferase activity (a "glyco-enzyme") to a cell derived from a primary tumor or a cell line. Applicants herein provide a methodology provide a method with which a disorder such as cancer may be treated by altering expression of a protein having sialyl- or glycosyltransferase activity, preferably α2,6-ST and / or α2,3-ST, within a cell. It is recognized by those skilled in the art that there is a need for methodologies with which to treat such disorders, as there is a lack of effective treatments resulting in the suffering and eventual death of many victims of such diseases. The invention of this application provides reagents and methodologies for treatment of a neurological disorder such as brain cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Glyco-Enzyme mRNA Expression in Gliomas.

Figure 2. Glyco-Enzyme mRNA Expression in Meningiomas.

Figure 3. Exemplary Glyco-Enzyme Nucleic Acid Constructs.

Figure 4. α2,3-ST expression in glioma specimens (panels A1 and A2) and brain metastases (panels B1 and B2). Panels A1 and A2. Lane 1: normal human brain; lanes 2-14: clinical glioma specimens; lane 15: U-373MG human glioma cell line. Panels B1 and B2. Lane 1: normal human brain; lanes 2-10: clinical specimens of brain metastases; lane 11: U-373MC human glioma cell line. All glioma specimens expressed α2,3-ST mRNA (panels A1) and seven out of nine metastases expressed α2,3-ST mRNA (panel B1). Ethidium bromide staining of total RNA is shown in panels A2 and B2.

Figure 5. Expression of α2,3-ST in human brain tumor cell lines and fetal astrocytes. All lanes were loaded with 20 μg total RNA. Panel 5A. Lanes 1-5: human

glioma cell lines SNB-19, SW1088, U-118MG, U-373MG, and U87MG, respectively; lanes 6-8: human neuroblastoma cell lines SKN-MC, LAN-S, and IMR 32, respectively. All brain tumor cell lines expressed α2,3-ST mRNA. Ethidium bromide staining of total RNA is shown in panel 5B. Panel 5C. Lane 1: human neuroblastoma IMR 32; lane 2: human neuroblastoma LAN-S; lane 3: cultured human fetal astrocytes; lane 4: human glioma U-373MG; lane 5: human glioma U-118MG. Ethidium bromide staining of total RNA is shown in panel 5D.

Figure 6. Increased *Maackia amurensis* agglutinin lectin (MAA) staining in gliomas. Surfaces of glioblastoma cells (A), extracellular matrices between glioblastoma cells (B) and glioblastoma parenchyma (C) were heavily stained, while vasculatures within the tumors (B, C) remained negative. Positive MAA staining was observed in capillaries of normal cerebral cortex, but not in neurons or glial cells (D). Bars = 50 μm.

Figure 7. Expression of α2,6-ST protein and α2,6-linked sialoglycoconjugates in transfected U373 MG cells. Transfected cells, prior to clonal selection, were grown on glass coverslips and immunofluorescence microscopy was performed as described in the Materials and Methods Section. The pcDNA3/~2,6-ST transfected cells (A, C, F) and pcDNA3 transfected cells (B, D, F) were stained with FITC-PHA-E (A, B) to detect bisecting type N-linked structures, anti- Q2,6-ST antibody (C, D), or FITC-SNA (F, F) to detect α2,6-linked sialoglycoconjugates.

Figure 8. Expression of α2,6-ST protein and α2,6-linked sialoglycoconjugates after subcloning. Fluorescence microscopy of clone #35. Clone #35cells were stained with anti-α2,6-ST antibody (A) or FITC-SNA (B). (C) and (D) are the corresponding phase contrast photomicrographs.

Figure 9. Expression of α2,6-ST mRNA and enzyme activity in U373 MG/α2,6-ST clones. Total RNA was isolated from parental glioma U373 MG cells, pcDNA3 transfected cells, and three pcDNA3/ α2,6-ST transfected clones (#18, #24, #35). 20 μg

of total RNA per lane was electrophoresed. The 1.45 kb rat α2,6 ST cDNA was used for Northern analyses (panel A). In order to assess α2,6-ST expression caused by transfection artifacts, pcDNA3-transfected U373 MG cells were used as a control. Panel A. Lane 1, parental U373MG cells; lane 2, U373 MG cells transfected with pcDNA3; lane 3, pcDNA3/α2,6-ST transfected clone #18; lane 4, clone #24; lane 5, clone #35. Total RNA staining by ethidium bromide is shown in panel B. Panel C. Relative α2,6-ST enzyme activity expressed by the three transfected clones. Enzyme activity was determined as described below. The data was normalized to the highest expressing clone, #35. α2,6-ST enzyme activity was not detected in the parental or in the pcDNA3 transfected cells.

Figure 10. ***In vitro*** **invasion assay of the U373 MG/α2,6ST transfectant.** Biocoat Matrigel Invasion Chambers (Collaborative Research, Bedford, MA) were used to evaluate the relative invasivity of the transfected subclones compared to pcDNA3 "mock" transfected controls. The data is an average of two separate experiments done in triplicate. Values did not vary by more than 10%.

Figure 11. ***In vitro*** **adhesion assay of the U373 MG/α2,6ST transfectant.** Human fibronectin or collagen type I coated 24-well plates were used to evaluate the relative adhesion of three transfectants (clones #18, #24, #35). Compared to a pcDNA3 "mock" transfected control, the transfectants showed a reduction in adhesion to both fibronectin substrate and collagen type I. These data are the average of three values taken from a representative experiment and did not vary by more than 10%.

Figure 12. α2,6-linked sialylation α3β1 integrin in the transfectant.

Figure 13. Adhesion-mediated protein tyrosine phosphorylation in the transfected clones.

Figure 14. Induction of focal adhesion kinase $p125^{fak}$ mRNA expression in α2,6ST

transfected U373 MG.

Figure 15. Adhesion-mediated protein tyrosine phosphorylation in the α2,3-ST and α2,6-ST clone. The α2,3-ST and α2,6-ST cells were incubated in fibronectin-coated flasks for 30 min, and unattached cells were removed by washing three times with cold PBS. The attached cells were then solubilized with 200 µl of lysis buffer. The lysate was centrifuged at 12,000 x g for 5 min to eliminate non-soluble material. 30 µg of protein from each sample were loaded on an 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane, and the membrane was incubated with 3% non-fat milk at 21°C for 30 min. Anti-phosphotyrosine antibody (Upstate Biotechnology) was then added at 1:1000 dilution and incubated at 21°C for 1 hr. The membrane was then washed three times with PBS containing 0.05% Tween 20, and the antibody-bound proteins were detected using an ECL kit (Amersham). A phosphorylated protein with a molecular mass of 110 kDa (arrow) was observed in α2,3-ST cells, but not in α2,6-ST transfected cells.

Figure 16. Morphological changes in cells transfected with pcDNA3 (control; 16A and 16B) or pcDNA3/a2,6ST (clone #18; 16C and 16D).

Figure 17. Cell Morphology of α2,6-ST transfected U-373MG glioma cells. α2,3-ST, α2,6-ST, vector-transfected control (pcDNA3) and parental U-373MG cells were grown to confluence on culture dish in DMEM containing 10% FBS, and cells were photographed.

Figure 18. Cell spreading in the α2,3-ST and α2,6-ST transfected cells. α2,3-ST, α2,6-ST, vector-transfected control (pcDNA3) and parental U-373MG cells were plated on culture dish in DMEM containing 10% FBS and incubated at 37°C to allow spreading on the plate. Cells were photographed at 1, 3, and 24 hrs. later.

Figure 19. Loss of tumorigenicity in α2,6-ST transfectants in the nude mouse hindflank model. Parental U-373MG cells and α2,6-ST transfected cells were implanted at the hindflank of the nude mouse to examine the effect of α2,6-ST expression on tumorigenicity.

Figure 20. α2,6-ST transfectants are not tumorigenic in the SCID mouse intracranial glioma model. 1.25 x 10^6 Glioma cells were injected stereotactically into the right basal ganglia of anesthetized SCID mice (C.B-17 scid/scid, 6 week-old, Charles River Lab). The brains were harvested at six weeks post injection and 6 μm sections were stained with hematoxylin and eosin (A-C) or anti-human EGF receptor antibody (D-F). A & D: pcDNA3 vector-transfected U-373MG, B & E: α2,6-ST gene transfected U-373MG cells, C & F: α2,3-ST gene transfected U-373MG cells. Tumor formation is shown by arrows. No measurable tumor is found in α2,6-ST gene transfected U-373MG; the needle tract is shown by arrows.

Figure 21. Effect of α2,6-ST gene transfection on intracranial tumor formation. Differences in tumor size between each group were compared to tumor size of the pcDNA3 vector-transfected U-373MG control group as 100%. Parental U-373MG glioma cells, three different α2,6-ST transfected U-373MG glioma clones (J11, J20, J22), three different α2,3-ST transfected U-373MG glioma clones (J8, J22 and J2), and pcDNA3 vector transfected U-373MG cells were used. Difference in tumor size among the animal groups were determined by chi-squared analysis

Figure 22. The expression of GnT-III and GnT-V mRNA in glioma specimens. 30 μg of total RNA per lane were used for Northern analysis. Lane 1: normal human brain. Lanes 2-14: clinical glioma specimens. Increased GnT-III expression is seen in lanes 3 and 10 compared to normal brain, while other specimens showed similar levels or less than that in normal brain (panel A). Enhanced GnT-V mRNA expression is seen in lanes 3, 4, 7 and 10 (panel B), and other samples showed similar levels or less compared to that in normal brain. Ethidium bromide staining of total RNA (panel C).

Figure 23. Expression of GnT-III, GnT-V, and c-ets-1 mRNA in human brain tumor cell lines. 20 μg of total RNA per lane were used for Northern analysis. Left panel: lanes 1-5 are human glioma cell lines, and lanes 6-9 are human neuroblastoma cell lines. Lane 10: Hep G2 human hepatocarcinoma as a positive control for GnT-III and GnT-V expression. Right panel: lanes 1-6 are human glioma cell lines, and lanes 7-10 are human neuroblastoma cell lines. All brain tumor cell lines expressed similar amounts of GnT-III mRNA (A), but GnT-V expression varied among the cell lines (B). Brain tumor cell lines with high GnT-V expression (D) also showed robust expression of c-ets-1 mRNA (E). Ethidium bromide staining of total RNA (C & F).

Figure 24. L-PHA lectin staining of human glioma specimens. L-PHA lectin staining showed variable but typical morphological features found in high-grade astrocytomas. Figure 2A shows the cell surface staining of a specimen of glioblastoma that characteristically contained zonal necrosis and multiple nucleated tumor cells. In another glioblastoma specimen (Fig. 2B), the lectin staining was found in extracellular matrices between undifferentiated small tumor cells with high cellularity. Cytoplasmic round bodies in gemistocytic astrocytoma cells were also stained with the lectin (Fig. 2C), while normal astrocytes were not stained (Fig. 2D). L-PHA-stained the vasculature found in the glioblastoma specimens, which was closely related to the distribution of tumor, but varied in size and shape: endothelial cells in capillaries, thin-walled vessels with extended lumina, thick-walled larger vessels, and vessels with convoluted lumina (glomeruloid vessels). These vessels were compatible morphologically with the well described neovascularization typically found in glioblastomas; the staining pattern was consistent with the idea that L-PHA binds to the vascular basement membrane produced by the glioblastoma cells. Bar = 20μm.

Figure 25. Expression of L-PHA binding proteins and Ets-1 protein in glioma cell lines. Panel A: L-PHA lectin was used to detect glycoproteins carrying β1,6-GlcNAc N-glycan. Panel B: Western blot of Ets-1 protein using monoclonal anti-Ets-1 antibody.

Lanes 1-5; human glioma cell lines, SW1088, U-118MG, U-373MG, U-87MG, and D-54MG, respectively. Lanes 6-9; human neuroblastoma cell lines, SKN-SH, SKN-MC, LAN-5 and IMR-32, respectively. L-PHA lectin recognized the 140 kDa glycoprotein (arrow) in all human glioma cell lines (A). 51 kDa Ets-1 protein was detected in all glioma and neuroblastoma cell lines (B).

Figure 26. Stable transfection of *GnT-V* gene into human glioma U-373MG cells. 20 μg of total RNA per lane were used for Northern analyses. Lane 1: parental U-373MG glioma cells, lane 2: pcDNA3 vector-transfected U-373MG, and lanes 3-7: GnT-V transfected U-373MG clones. GnT-V stable transfectants express the 3.0 kb GnT-V transcript in addition to the endogenous 9.5 kb transcript (arrow, Panel A). Ethidium bromide staining of total RNA (Panel B).

Figure 27. Cell morphology of GnT-transfected clones. Phase-contrast photomicrographs of U-373MG cells (A), GnT-III transfected cells (B), GnT-V transfected cells (C), and pcDNA3 vector-transfected cells (D). Parental U-373MG cells show similar cell morphology with the vector-transfected control cells. GnT-V transfected cells have fan-shaped cell morphology with a distinct leading edge, while GnT-III transfected cells are well-spread.

Figure 28. Immunofluorescence microscopy of GnT-transfected cells using monoclonal antibodies against α3β1 integrin and vinculin. Photomicrographs A, C, E, and G are cells stained with anti-vinculin antibody. Photomicrographs B, D, F, and H are cells stained with anti-α3β1 integrin antibody. U-373MG cells (A & B), GnT-III transfected cells (C & D), GnT-V transfected cells (E & F), and pcDNA3 vector-transfected controls (G & H).

Figure 29. *In Vitro* invasion assays of GnT-V transfectants. The relative invasivity of GnT-V transfected U-373MG clones was compared with the invasivity of pcDNA3 vector-transfected U-373MG cells (lane 7) as 100%. Lanes 1-5: GnT-V transfected U-

373MG clones, lane 6: parental U-373MG cells, and lane 7: pcDNA3 vector-transfected U-373MG. The transfectants were 2-5 fold more invasive than the vector-transfected control cells and 4-10 fold more invasive than parental U-373MG cells. The levels of GnT-V mRNA expression in the transfected clones are mostly, but not always, correlated with the levels of invasivity. The data are the average ± SEM (bars) values of two separate experiments done in triplicate

Figure 30. Inhibition of glioma cell migration on fibronectin substrate by *Phaseolus vulgaris* isolectins. **A.** Two μg/ml of E-PHA strongly inhibited cell migration of parental U-373MG cells on a fibronectin substratum, and completely abolished the migration of the transfectants. The inhibitory effect was similar with 10 μg/ml of monoclonal anti-α3 integrin antibody (Chemicon, clone P1B5), while L-PHA showed little effect. **B.** Two μg/ml of E-PHA also inhibited cell migration of D-54MG, SNB-19, SW1088, and U-87MG glioma cells. At the same concentration, L-PHA showed little effect on cell migration. The data are average ± SD (bars) values of two separate experiments done in triplicate.

Figure 31. Construction of α2,6-ST Ad vector. A. pCMV-general ("pCMV-G") for generation of α2,6 Ad vector. **B.** Ad5 wild type vector. **C.** Adα2,6ST59 vector.

Figure 32. Adenovirus-mediated gene expression of α2,6-sialyltransferase in U373MG glioma cells. Replication-deficient Adenovirus (200 pfu/cell) was used to express α2,6-sialyltransferase mRNA in the glioma cells. Lane 1: A stable transfectant of α2,6-sialyltransferase gene in U373MG glioma cells (clone J20) which express a 2.1kb transcript; Lanes 2&3: Parental U373MG cells; Lanes 4&5: The Adeno/α2,6-ST virus-infected U373MG cells after 48hrs of incubation; Lanes 6&7: U373MG cells transiently transfected with the Adeno/α2,6-ST gene. Northern analysis was performed using a 1.6kb rat α2,6-sialyltransferase cDNA. 20 μg per lane of total RNA was used. As shown in this figure, Adeno/α2,6-ST virus-mediated gene expression is much higher than stable transfection or transient transfection.

Figure 33. Dose-dependent expression of α2,6-ST mRNA by Northern analysis. Lane 1:U-373MG cells 48 hrs following infection with crude Adα2,6ST59 virus, lane 2: U-373MG cells with no virus, Lanes 3-8: U-373MG cells 48 hrs following infection with, respectively, 0.02, 0.2, 2.0, 10.0, 20.0, and 200 plaque-forming units (pfu) /cell of purified Adα2,6ST59. 10 μg of total RNA per lane were used. A 2.1 kb α2,6-ST transcript was expressed (upper panel). Ethidium bromide staining of total RNA (lower panel).

Figure 34. Time-dependent expression of α2,6-ST mRNA by Northern analysis. Lane 1:U-373MG cells stably transfected with α2,6-ST gene. Lane 2: U-373MG cells with no virus, lanes 3-12: U-373MG cells infected with 10 pfu/cell Adα2,6ST59 virus at 3 hrs, 6 hrs, 12 hrs, 1 day, 2 days, 3 days, 4 days, 6 days, 7 days and 8 days post-infection, respectively. 10 μg of total RNA per lane were used. A 2.1 kb α2,6-ST transcript was expressed (upper panel). Ethidium bromide staining of total RNA (lower panel).

Figure 35. Time-dependent expression of α2,6-linked sialic acids by *Sambucus nigra* agglutinin (SNA) lectin Western blot analysis. Lane assignment is identical to Figure 26. 10 μg of whole cell lysate protein was applied onto an 8% SDS-PAGE gel, transferred to a PDVF membrane and stained with the lectin.

Figure 36. Adα2,6ST59 virus infection results in changes in cell morphology. Phase-contrast photomicrograghs of U-373MG glioma cells (A), AdCMV 2 infected U-373MG cells (B) and Adα2,6ST59 infected U-373MG cells (C). Parental U-373MG cells show similar cell morphology to AdCMVβ2 infected U-373MG cells, while the Adα2,6ST59 infected U-373MG cells show well-spread cell morphology with dendritic processes. Cells were infected with either virus at 10 pfu/cell and the photomicrograghs were taken at 48 hrs post infection.

Figure 37. Increased expression of p125fak mRNA in U-373MG cells infected with the Adα2,6ST59 virus. Northern blot probed with p125fak cDNA (panel A). Panel B shows the same blot probed with α2,6-ST cDNA probe. Ethidium bromide staining (panel C). Lane 1: U-373MG cells with no virus, lanes 2-8: U- 373MG cells infected with 10 pfu/cell Adα2,6ST59 virus, 1 day, 2 days, 3 days, 4 days, 6 days, 7 days and 8 days post-infection, respectively. 10 μg of total RNA per lane were used.

Figure 38. Inhibition of U-373MG glioma cell invasion by the Adα2,6ST59 virus. U-373MG glioma cells were infected with the Adα2,6ST59 virus (gray bars) or a control virus, AdCMVβ2 (black bars). Cells were infected at 1, 2, 5, 10 and 40 pfu/cell and maintained for an additional 4 days in culture. Cells were then used for an *in vitro* invasion assay according to methods described previously. Data is shown as percent invasion of parental U-373MG cells without virus infection. The data are the average ± SEM (bars) values of two separate experiments done in triplicate.

SUMMARY OF THE INVENTION

The present invention provide reagents and methodologies for treatment a disease condition in which a glyco-enzyme has a role. As an example of such a disease condition, Applicants have demonstrated the reagents and methodologies of the present invention using a neurological disease model. Many neurological disorders such as brain cancer, Parkinson's disease and Alzheimer's disease are associated with a poor prognosis. Options for treatment of these diseases is currently extremely limited. The present invention provides a reagents and methodologies with which such a prognosis may be improved. The present invention provides reagents and methodologies for treating and preventing diseases in which alterations in the sialyation and/or glycosylation of proteins are involved.

In one embodiment of the present invention, a method of treating a neurological disorder comprising transfection of an isolated nucleic acid molecule encoding a protein having sialyl- or glycosyltransferase ("glyco-enzyme") activity into a target cell.

Preferably, expression of the protein having such activity within the target cell decreases the ability of that cell to proliferate or function or increases the ability of the host immune system to recognize the target cell. More preferably, and due to any of multiple possible mechanisms, the target cell is unable to survive following expression of the protein. Preferably, the sialyl- or glycosyltransferase (i.e., glyco-enzyme) is α2,6-ST, α2,3-ST, SLex-ST, Fuco, HexB, GnTI, GnTIII, and GnTV.

In another embodiment of the present invention, a viral vector comprising a nucleic acid encoding a glyco-enzyme protein is provided. In another embodiment, a method for treating a neurological disorder using a viral vector such as that described above is provided. Preferably, the glyco-enzyme is α2,6-ST, α2,3-ST, SLex-ST, Fuco, HexB, GnTI, GnTIII, and GnTV.

Many other embodiments will be understood by the skilled artisan to be within the scope of the instant invention, as further described in this application.

DETAILED DESCRIPTION

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references including: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.). The term "glyco-enzyme" is to be understood to refer to a sialyl- or glycosyltrans-ferase.

The types and amounts of glyco-enzymes found within neural tissues varies signficantly. Applicants have previously reported that α2,6-ST is expressed in a wide variety of normal human and rat tissues, including the skin, hematopoietic tissues, esophagus, liver, kidney, uterus and placenta (Kaneko, 1995). In addition, that study demonstrated α2,6-ST expression in normal choroid plexus epithelial and ependymal

cells of the nervous system (Kaneko, 1995). α2,3-ST has been shown to be expressed primarily in skeletal muscle, brain and most fetal tissues (Kitagawa, 1994). The variation of mRNA expression of glyco- enzymes is demonstrated in Figures 1 and 2. As indicated in **Figure 1**, the majority of gliomas tested express low levels of α2.6-ST, Fuco, GnTI and GnTV but express higher levels of α2,3-ST, SLex-ST, HexB, and GnTIII. In order to alter the patterns of glycosylation or sailylation on such cells, the present invention provides the reagents and methodologies for mainipulating the levels of these enzymes may be manipulated by introduction of nucleic acid molecules or other agents that direct or inhibit expression of such enzymes.

Applicants have also studied the expression of glyco-enzymes in meningiomas (**Figure 2**). The data indicate that the majority of meningiomas tested express relatively high levels of α2,6-ST, α2,3-ST, Fuco, and GnTIII; relatively moderate levels of HexB and GnTI; and relatively low levels of SLex-ST and GnTV. The reagents and methodologies of the present invention may be utilized to alter glycosylation and sialylation in meningioma by, for instance, transfecting into a meningioma an antisense construct. Similarly, introduction of an expression vector encoding a glyco-enzyme such as that provided by the present invention into a meningioma provides an increased amount of enzyme in the cell resulting in alteration of glycosylation patterns. Either of the above methodologies will decrease tumorigenicity by, for example, decreasing adhesivity or increasing immunogenicity.

In practicing the present invention, it is advantageous to transfect into a cell a nucleic acid construct directing expression of a protein or nucleic acid product having the ability to alter expression of a glyco-enzyme. There are available to one skilled in the art multiple viral and non-viral methods suitable for introduction of a nucleic acid molecule into a target cell. Genetic manipulation of primary tumor cells has been described previously (Patel et al., 1994). Genetic modification of a cell may be accomplished using one or more techniques well known in the gene therapy field (Human Gene Therapy April 1994, Vol. 5, p. 543-563; Mulligan, R.C. 1993). Viral transduction methods may comprise the use of a recombinant DNA or an RNA virus comprising a nucleic acid sequence that drives or inhibits expression of a protein having sialyltransferase activity to

infect a target cell. A suitable DNA virus for use in the present invention includes but is not limited to an adenovirus (Ad), adeno-associated virus (AAV), herpes virus, vaccinia virus or a polio virus. A suitable RNA virus for use in the present invention includes but is not limited to a retrovirus or Sindbis virus. It is to be understood by those skilled in the art that several such DNA and RNA viruses exist that may be suitable for use in the present invention.

Adenoviral vectors have proven especially useful for gene transfer into eukaryotic cells (Stratford-Perricaudet and Perricaudet. 1991). Adenoviral vectors have been successfully utilized to study eukaryotic gene expression (Levrero, M., et al. 1991). vaccine development (Graham and Prevec, 1992), and in animal models (Stratford-Perricaudet, et al. 1992.; Rich, et al. 1993). The first trial of Ad-mediated gene therapy in human was the transfer of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to lung (Crystal, et al., 1994). Experimental routes for administrating recombinant Ad to different tissues *in vivo* have included intratracheal instillation (Rosenfeld, et al. 1992) injection into muscle (Quantin, B., et al. 1992), peripheral intravenous injection (Herz and Gerard, 1993) and stereotactic inoculation to brain (Le Gal La Salle, et al. 1993). The adenoviral vector, then, is widely available to one skilled in the art and is suitable for use in the present invention.

Adeno-associated virus (AAV) has recently been introduced as a gene transfer system with potential applications in gene therapy. Wild-type AAV demonstrates high-level infectivity, broad host range and specificity in integrating into the host cell genome (Hermonat and Muzyczka. 1984). Herpes simplex virus type-1 (HSV-1) is attractive as a vector system for use in the nervous system because of its neurotropic property (Geller and Federoff. 1991; Glorioso, et al. 1995). Vaccinia virus, of the poxvirus family, has also been developed as an expression vector (Smith and Moss, 1983; Moss, 1992). Each of the above-described vectors are widely available to one skilled in the art and would be suitable for use in the present invention.

Retroviral vectors are capable of infecting a large percentage of the target cells and integrating into the cell genome (Miller and Rosman. 1989). Retroviruses were developed as gene transfer vectors relatively earlier than other viruses, and were first used

successfully for gene marking and transducing the cDNA of adenosine deaminase (ADA) into human lymphocytes.

It is also possible to produce a viral vector in vivo by implantation of a "producer cell line" in proximity to the target cell population. As demonstrated by Oldfield, et al. (1993), infiltration of a brain tumor with cells engineered to produce a viral vector carrying an effector gene results in the continuous release of the viral vector in the vacinity of the tumor cells for an extended period of time (i.e, several days). In such a system, the vector is retroviral vector which preferably infects proliferating cells, which, in the brian, would include mainly tumor cells. The present invention provides a methodology with which a viral vector supplies a nucleic acid sequence encoding a protein having sialyl- or glycosyl transferase activity to cells involved in a nuerological disorder such as brain cancer.

"Non-viral" delivery techniques that have been used or proposed for gene therapy include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA, $CaPO_4$ precipitation, gene gun techniques, electroporation, and lipofection (Mulligan, 1993). Any of these methods are widely available to one skilled in the art and would be suitable for use in the present invention. Other suitable methods are available to one skilled in the art, and it is to be understood that the present invention may be accomplished using any of the available methods of transfection. Several such methodologies have been utilized by those skilled in the art with varying success (Mulligan, R. 1993). Lipofection may be accomplished by encapsulating an isolated DNA molecule within a liposomal particle and contacting the liposomal particle with the cell membrane of the target cell. Liposomes are self-assembling, colloidal particles in which a lipid bilayer, composed of amphiphilic molecules such as phosphatidyl serine or phosphatidyl choline, encapsulates a portion of the surrounding media such that the lipid bilayer surrounds a hydrophilic interior. Unilammellar or multilammellar liposomes can be constructed such that the interior contains a desired chemical, drug, or, as in the instant invention, an isolated DNA molecule.

The cells may be transfected in vivo (preferably at the tumor site), ex vivo (following removal from a primary or metastatic tumor site), or in vitro. The cells may

be transfected as primary cells isolated from a patient or a cell line derived from primary cells, and are not necessarily autologous to the patient to whom the cells are ultimately administered. Following ex vivo or in vitro transfection, the cells may be implanted into a host, preferably a patient having a neurological disorder and even more preferably a patient having a brain tumor. Genetic manipulation of primary tumor cells has been described previously (Patel et al. 1994). Genetic modification of the cells may be accomplished using one or more techniques well known in the gene therapy field (Human Gene Therapy. April 1994. Vol. 5, p. 543-563; Mulligan, R.C. 1993).

In order to obtain transcription of the nucleic acid of the present invention within a target cell, a transcriptional regulatory region capable of driving gene expression in the target cell is utilized. The transcriptional regulatory region may comprise a promoter, enhancer, silencer or repressor element and is functionally associated with a nucleic acid of the present invention. Preferably, the transcriptional regulatory region drives high level gene expression in the target cell. It is further preferred that the transcriptional regulatory region drives transcription in a cell involved in a neurological disorder such as brain cancer. Transcriptional regulatory regions suitable for use in the present invention include but are not limited to the human cytomegalovirus (CMV) immediate-early enhancer/promoter, the SV40 early enhancer/promoter, the JC polyomavirus promoter and the chicken β-actin promoter coupled to the CMV enhancer (Doll, et al. 1996).

The vectors of the present invention may be constructed using standard recombinant techniques widely available to one skilled in the art. Such techniques may be found in common molecular biology references such as *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), and *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA). Examples of nucleic acid constructs useful for practicing the present invention comprise a transcriptional regulatory region such as the CMV immediate-early enhancer/promoter, the SV40 early enhancer/promoter, the JC polyomavirus promoter, or the chicken β-actin promoter

coupled to the CMV enhancer operably linked to a nucleic acid encoding a glyco-enzyme.

In practicing the present invention, the glyco-enzyme is preferably α2,6-ST (i.e., GenBank L29554; SEQ ID NO.: 19; α2,6-ST polypeptide encoded by nucleotides 226-1143 of SEQ ID NO. 19 is shown in SEQ ID NO. 20); α2,3-ST (i.e., GenBank Accession No. L23768; SEQ ID NO.: 5; α2,3-ST polypeptide encoded by nucleotides 1 – 1128 of SEQ ID NO.:5 is shown in SEQ ID NO.: 6); SLex-ST (i.e., GenBank No. X74570; SEQ ID NO.: 11; Slex-ST polypeptide encoded by 163-1152 of SEQ ID NO. 11 is shown in SEQ ID NO.: 12), Fuco (i.e., GenBank NM_000147; SEQ ID NO.: 9; Fuco polypeptide encoded by nucleotides 19-1404 of SEQ ID NO.: 9 is shown in SEQ ID NO.: 10); HexB (i.e., GenBank Accession No. NM_000521; SEQ ID NO. 7; HexB polypeptide encoded by nucleotides 76-1746 of SEQ ID NO.: 7 shown in SEQ ID NO.: 8); GnTI (i.e., GenBank Accession No. NM_002406; SEQ ID NO. 13; GnTI polypeptide encoded by nucleotides 497-1834 of SEQ ID NO.: 13 shown in SEQ ID NO.: 14); GnTIII (i.e., GenBank Accession No. NM_002409; SEQ ID NO. 15; GnTIII polypeptide encoded by nucleotides 247-1842 of SEQ ID NO.: 15 shown in SEQ ID NO.: 16) or GnTV (i.e., GenBank Accession No. D17716; SEQ ID NO. 17; GnTV polypeptide encoded by nucleotides 146-2371 of SEQ ID NO.: 17 shown in SEQ ID NO.: 18). Other suitable glyco-enzymes are known to those of skill in the art and fall within the scope of the present invention.

To generate such a construct, a nucleic acid sequence encoding the enzyme may be processed using one or more restriction enzymes such that certain sequences flank the nucleic acid. Processing of the nucleic acid may include the addition of linker or adapter sequences. A nucleic acid sequence comprising a preferred transcriptional regulatory region may be similarly processed such that the sequence has flanking sequences compatible with the nucleic acid sequence encoding the enzyme. These nucleic acid sequences may then be joined into a single construct by processing of the fragments with an enzyme such as DNA ligase. The joined fragment, comprising a transcriptional regulatory region operably linked to a nucleic acid encoding a glyco-enzyme, may then be inserted into a plasmid capable of being replicated in a host cell by further processing

using one or more restriction enzymes. Exemplary vector constructions are illustrated in **Figure 3**.

Administration of a nucleic acid of the present invention to a target cell in vivo may be accomplished using any of a variety of techniques well known to those skilled in the art. Such reagents may be administered by intravenous injection or using a technique such as stereotactic injection to administer the reagent into the target cell or the surrounding areas (Badie, et al. 1994; Perez-Cruet, et al. 1994; Chen, et al. 1994; Oldfield, et al. 1993; Okada, et al. 1996).

The vectors of the present invention may be administered orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intramuscular, intrasternal, infusion techniques or intraperitoneally. Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable non-irritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum and release the drug.

The dosage regimen for treating a neurological disorder disease with the vectors of this invention and/or compositions of this invention is based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely using standard methods.

The pharmaceutically active compounds (i.e., vectors) of this invention can be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, including humans and other mammals. For oral administration, the pharmaceutical composition may be in the form of, for example, a capsule, a tablet, a suspension, or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a given amount of DNA or viral vector particles (collectively referred to as "vector"). For example, these may contain an amount of vector from about 10^3-10^{15} viral particles, preferably from about 10^6-10^{12} viral particles.

A suitable daily dose for a human or other mammal may vary widely depending on the condition of the patient and other factors, but, once again, can be determined using routine methods. The vector may also be administered by injection as a composition with suitable carriers including saline, dextrose, or water.

Injectable preparations, such as sterile injectable aqueous or oleaginous suspensions, may be formulated according to the known are using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

A suitable topical dose of active ingredient of a vector of the present invention is administered one to four, preferably two or three times daily. For topical administration, the vector may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation. Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The pharmaceutical compositions may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The pharmaceutical compositions may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc. Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert diluent such as sucrose, lactose, or starch. Such dosage forms may also

comprise, as in normal practice, additional substances other than inert diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings. Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting sweetening, flavoring, and perfuming agents.

While the nucleic acids and /or vectors of the invention can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more vectors of the invention or other agents. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

The present invention may comprise elevation or depression of enzyme levels in cells expressing various amounts of enzyme. Introduction of an glyco-enzyme expression vector into a cell already expressing a high level of that enzyme may alter glycosylation patterns within that cell. Similarly, introduction of a nucleic acid construct that inhibits expression of such an enzyme in a cell expressing low levels of that enzyme may also serve to alter glycosylation patterns in that cell. Either of these methodologies may decrease the tumorigenicity or a malignancy of the cell.

The reagents and methodologies of the present invention may be utilized to treat or prevent a variety of disorders in which glycosylation is involved. An example of such a disorder is cancer. Cancer is defined herein as any cellular malignancy for which a loss of normal cellular controls results in unregulated growth, lack of differentiation, and increased ability to invade local tissues and metastasize. Cancer may develop in any tissue of any organ at any age. Cancer may be an inherited disorder or caused by environmental factors or infectious agents; it may also result from a combination of these. For the purposes of utilizing the present invention, the term cancer includes both neoplasms and premalignant cells.

In one embodiment, the present invention relates to the treatment or detection of brain cancer. Brain cancer is defined herein as any cancer involving a cell of neural origin. Examples of brain cancers include but are not limited to intracranial neoplasms such as those of the skull (i.e., osteoma, hemangioma, granuloma, xanthoma, osteitis deformans), the meninges (i.e., meningioma, sarcoma, gliomatosis), the cranial nerves (i.e., glioma of the optic nerve, schwannoma), the neuroglia (i.e., gliomas) and ependyma (i.e., ependymomas), the pituitary or pineal body (i.e., pituitary adenoma, pinealoma), and those of congenital origin (i.e., craniopharygioma, chordoma, germinoma, teratoma, dermoid cyst, angioma, hemangioblastoma) as well as those of metastatic origin. In certain embodiments, the preferred brain cancer cell is a glioma or a meningioma cell.

In one embodiment of the present invention, a method for decreasing the tumorigenicity or malignancy of a brain cancer cell comprising altering the expression of glycosylation of proteins produced by said cell, wherein the altered pattern of glycosylation is caused by the alteration of activity of one or more glyco-enzymes within said cell is provided. Alteration of activity may be accomplished by either inhibiting the activity of the glyco-enzyme directly using, for example, a binding agent such as an antibody, or indirectly using a nucleic acid or other agent that inhibits transcription or translation of the nucleic acid encoding the glycosyltransferase. Preferably, the glyco-enzyme is selected from α2,3-ST glycosyltransferase, α2,6-ST glycosyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase or GnTV glycosyltransferase.

In one embodiment, the activity of α2,3-ST sialyltransferase α2,6-ST sialyltransferase or GnTIII glycosyltransferase, or GnTV glycosyltransferase is altered. In a preferred embodiment, the activity of α2,3-ST sialyltransferase, α2,6-ST sialyltransferase or GnTIII glycosyltransferase is increased over normal levels in the cell. In another preferred embodiment, the activity of GnTV glycosyltransferase is decreased over normal levels in the cell.

In one embodiment, the present invention provides a methodology for transfection of a nucleic acid sequence, preferably an antisense oligonucleotide or polynucleotide, that inhibits expression or activity of a glyco-enzyme within a cell. A suitable oligonucleotide

may be designed using techniques that are well known in the field, such as an oligonucleotide that is complementary to the coding sequence of a glycosyltransferase. One example of a suitable antisense oligonucleotide comprises a functional nucleotide sequence such as a 2',5'-oligoadenylate as described in U.S. Patent No. 5,583,032. Using an antisense oligonucleotide, expression of the glyco-enzyme may be inhibited by inhibition of transcription, destruction of the transcript encoding the protein or inhibition of translation of the protein from its transcript. Inhibition of glyco-enzyme activity may be caused by the hybridization of an anti-sense DNA polynucleotide specific to a target nucleic acid encoding for or involved in the expression of a glyco-enzyme. In one embodiment, the target nucleic acid sequence hybridizes to a nucleic acid selected from a nucleic acid encoding α2,3-ST glycosyltransferase, α2,6-ST glycosyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase or GnTV glycosyltransferase. In a preferred embodiment, the target nucleic acid sequence encodes the GnTV glycosyltransferase. The resultant decrease in expression of these enzymes results in altered patterns of glycosylation, and, as described above, decreases tumorigenicity of the cancer cell.

As mentioned above, alteration of the activity of a glyco-enzyme may also be caused by the increase of activity of a glyco-enzyme within a cell. Preferably, the glyco-enyme is selected from α2,3-ST sialyltransferase α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase or GnTV glycosyltransferase. In a preferred embodiment, the glyco-enzyme is α2,3-ST, α2,6-ST or GnTIII glycosyltransferase. In a more preferred embodiment, the glycosyltransferase is α2,6-ST glycosyltransferase.

In one embodiment, the increased activity of a glyco-enzyme is caused by transfection of an exogenous DNA encoding for a glyco-enzyme, expressibly linked to a transcriptional regulatory region or promoter, into a cell wherein the exogenous DNA encodes α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST

glycosyltransferase or GnTV glycosyltransferase. In a preferred embodiment, the activity of α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, or GnTIII glycosyltransferase is increased.

In another embodiment, the present invention provides an isolated nucleic acid sequence encoding for a recombinant, replication-deficient adenovirus comprising a nucleic acid encoding a glycosyltransferase. Preferably, the glyco-enzyme is selected from α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase or GnTV glycosyltransferase. And, in yet another embodiment, an isolated nucleic acid sequence encoding for a recombinant, the glycosyltransferase-encoding nucleic acid sequence is under the transcriptional control of a regulator selected from the group consisting of CMV immediate-early enhancer/promoter, SV40 early enhancer/promoter, JC polyomavirus promoter, and chicken β-actin promoter is provided.

In another embodiment, the present invention provides a recombinant adenoviral particle containing a nucleic acid encoding for a glycosyltransferase such as α2,3-ST glycosyltransferase, α2,6-ST glycosyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase and GnTV glycosyltransferase. In yet another embodiment, the expression of the nucleic acid encoding for the glyco-enzyme is under transcriptional control of a regulator selected from the group consisting of CMV immediate-early enhancer/promoter, SV40 early enhancer/promoter, JC polyomavirus promoter, and chicken β-actin promoter. In certain embodiments of the present invention, transfection of a cell is performed.

Transfection may be performed using any suitable transfection method, many of which are well known in the art. Such methods may include, for example, calcium phosphate, liposomes, electroporation, or vector-assisted methods. In a preferred embodiment, the cell is involved in the causation of a neurological disorder such as brain cancer, Parkinson's disease or Alzheimer's disease. In a preferred embodiment, the cell is a cancer cell, and in a more preferred embodiment, the cell is a brain cancer cell. In certain embodiments, the present invention includes the transfer of a nucleic acid

glycosyltransferase or GnTV glycosyltransferase. In a preferred embodiment, the activity of α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, or GnTIII glycosyltransferase is increased.

In another embodiment, the present invention provides an isolated nucleic acid sequence encoding for a recombinant, replication-deficient adenovirus comprising a nucleic acid encoding a glycosyltransferase. Preferably, the glyco-enzyme is selected from α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase or GnTV glycosyltransferase. And, in yet another embodiment, an isolated nucleic acid sequence encoding for a recombinant, the glycosyltransferase-encoding nucleic acid sequence is under the transcriptional control of a regulator selected from the group consisting of CMV immediate-early enhancer/promoter, SV40 early enhancer/promoter, JC polyomavirus promoter, and chicken β-actin promoter is provided.

In another embodiment, the present invention provides a recombinant adenoviral particle containing a nucleic acid encoding for a glycosyltransferase such as α2,3-ST glycosyltransferase, α2,6-ST glycosyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase and GnTV glycosyltransferase. In yet another embodiment, the expression of the nucleic acid encoding for the glyco-enzyme is under transcriptional control of a regulator selected from the group consisting of CMV immediate-early enhancer/promoter, SV40 early enhancer/promoter, JC polyomavirus promoter, and chicken β-actin promoter. In certain embodiments of the present invention, transfection of a cell is performed.

Transfection may be performed using any suitable transfection method, many of which are well known in the art. Such methods may include, for example, calcium phosphate, liposomes, electroporation, or vector-assisted methods. In a preferred embodiment, the cell is involved in the causation of a neurological disorder such as brain cancer, Parkinson's disease or Alzheimer's disease. In a preferred embodiment, the cell is a cancer cell, and in a more preferred embodiment, the cell is a brain cancer cell. In certain embodiments, the present invention includes the transfer of a nucleic acid

sequence encoding a protein having the ability to add a glycosyl moiety (i.e., glycosyltransferase) to a substrate protein. Preferably, the nucleic acid sequence encodes a glyco-enzyme. More preferably, the nucleic acid encodes or more of α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, SLeX-ST glycosyltransferase, Fuco, HexB, GnTI glycosyltransferase, GnTIII or GnTV glycosyltransferaese. Even more preferably, the nucleic acid comprises a sequence encoding a glyco-enzyme that is under the transcriptional control of a transcriptional regulatory region which functions within a neural tissue or cell.

For instance, in certain embodiments of the present invention, a nucleic acid molecule encoding a α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase or GnTV glycosyltransferase and being under the transcriptional control of a transcriptional regulatory region that functions in a cancer cell is transfected into the cancer cell. This results in increased expression of the encoded enzyme resulting in altered glycosylation patterns of cellular proteins resulting in decreased tumorigenicity or malignancy by, for example, altering the adhesive potential or immunogenicity of the cell.

In another embodiment of the present invention, a target cell is transfected in vivo by implantation of a "producer cell line" in proximity to the target cell population (Culver, et al. 1994; Oldfield, 1993). The producer cell line is engineered to produce a viral vector and releases viral particles in the vicinity of the target cell. A portion of the released viral particles contact the target cells and infect those cells, thus delivering a nucleic acid of the present invention to the target cell. Following infection of the target cell, expression of the product of nucleic acid of the present invention occurs. Preferably, expression results in either increased or decreased expression of a protein having glycosyltransferase or sialyltransferase activity. More preferably, the protein is α2,6-ST sialyltransferase; α2,3-ST sialyltransferase; SLex-ST glycosyltransferase; Fuco glycosyltransferase; HexB sialyltransferase; GnTI sialyltransferase; GnTIII sialyltransferase or GnTV sialyltransferase.

The present invention further provides a method for detecting the tumorigenicity or malignancy of a brain cell, comprising measuring the expression of glycosyltransferase within said cell. Any method for detection of the glycosyltransferase may be utilized including but not limited to assays for the presence or activity of the glycosyltransferase protein within a cell or assays for detecting nucleic acids encoding or involved in the expression of a glycosyltransferase. Detection of a nucleic acid encoding a glycosyltransferase may be accomplished by detection of glycosyltransferase mRNA using any of several techniques available to one skilled in the art such as northern blot (Alwine, et al. Proc. Natl. Acad. Sci. 74:5350), RNase protection (Melton, et al. Nuc. Acids Res. 12:7035), or RT-PCR (Berchtold, et al. Nuc. Acids. Res. 17:453).

Detection of nucleic acids may be accomplished by hybridizing nucleic acids or polynucleotides to one another, and detecting the hybidized product which may include a nucleic acid or polynucleotide labelled with a detectable label. A nucleic acid or "polynucleotide" of the present invention includes those polynucleotides capable of hybridizing, under stringent hybridization conditions, to a nucleic acid encoding a glycosyltransferase of the present invention, or the complement thereof, or the cDNA. "Stringent hybridization conditions" refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency), salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH_2PO_4; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 ug/ml salmon sperm blocking DNA; followed by washes at 50°C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility. It is also possible to utilize commercial hybridization systems such as EXPRESS HYB (Stratagene, La Jolla, CA). Other modifications of such conditions are known to those of skill in the art and contemplated to be encompassed by the present invention.

In one embodiment, the glyco-enzyme is selected from the group consisting of α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase and GnTV glycosyltransferase. In another embodiment, detection of expression of the glyco-enzyme is accomplished by detection of nucleic acid sequences encoding the glyco-enzyme.

In yet another embodiment, the present invention comprises a kit for determining the tumorigenicity or malignancy of a brain cell. The kit may comprise a panel of independent or paired nucleic acid molecules specific for the detection of the expression of specific nucleic acid sequences corresponding to specific species of glycosyltransferase. One embodiment of such a kit utilizes enzyme-mediated nucleic acid amplification such as the polymerase chain reaction (PCR) in which a pair of nucleic acid molecules (i.e., primers) that allow for amplification of a nucleic acid sequence encoding α2,3-ST sialyltransferase, α2,6-ST sialyltransferase, HexB glycosyltransferase, Fuco glycosyltransferase, GnTIII glycosyltransferase, GnTI glycosyltransferase, SLex-ST glycosyltransferase and GnTV glycosyltransferase. As illustrated in **Figures 1** and **2**, the levels of expression of glyco-enzymes differs in various tumor types and a kit allowing for determining such levels of expression may be utilized to predict or determine tumorigenicity of certain cell samples.

Detection of expression of a glyco-enzyme within a cell also provides for the identification of compounds or other treatment modalities useful for treating a disorder. For instance, a compound may be applied to a brain cancer cell line and the levels of glyco-enzyme expression determined. Compounds that either increase or decrease expression of the glyco-enzyme are candidates for treatment of a disorder in which glyco-enzymes play a role. In one embodiment, a compound may be shown to increase expression of α 2,6-ST, thus inhibiting tumorigenicity or malignancy of the cells.

Using these methods, it is also possible to "customize" a treatment protocol to a particular patient. For instance, a brain tumor or portion thereof is removed from a patient and a single cell suspesion or similar culture prepared. The cells are then exposed to a potential chemotherapeutic agent or other therapeutic such as radiation to measure glyco-enzyme expression. On the one hand, compounds or treatments that alter glyco-enzyme expression may be useful to treat the tumor (i.e., if the compound increases α 2, 6-ST expression in a brain tumor). On the other hand, compounds or treatments that decrease expression of other enzymes such as GnTV may be useful. Using these methods, compounds or treatment modalities that would not provide optimal benefit to the patient may be avoided.

The following Examples are for illustrative purposes only and are not intended, nor should they be construed, as limiting the invention in any manner. Those skilled in the art will appreciate that variations and modifications can be made without violating the spirit or scope of the invention.

EXAMPLES

The experiments presented herein demonstrate that alterations in the expression of normal cell-surface carbohydrates can modulate the invasive potential of malignant gliomas. Unless otherwise stated, all established human brain tumor cell lines utilized in these examples were maintained using Dulbecco's modified Eagle's medium (DMEM, containing 4.5 g/L glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Whittaker BioProducts, Walkersville, MD). The following cell lines were used for Northern analysis: Human glioblastoma, SNB-19 and D-54MG (generously provided by

Dr. Paul Kornblith, Univ. of Pittsburgh and Dr. Darrell Bigner, Duke University, respectively); Human glioblastomas, U-87MG, U-373MG, U-118MG, and SW1088 (American Type Culture Collection (ATCC), Rockville, MD); Human neuroblastoma cell lines, SKN-SH, SKN-MC and IMR 32 (ATCC), and LAN-5 (generously provided by Dr. Stephan Ladish, Children's Research Institute, Washington DC); Human hepatocarcinoma, Hep G2 (ATCC) as a positive control for GnT-III and GnT-V. For Northern analysis of GnT-III and GnT-V, a panel of surgical specimens was used that consisted of 13 gliomas: 1 astrocytoma grade II, 1 high-grade oligodendroglioma, 1 mixed glioma, 3 cases of astrocytoma grade III and 7 cases of astrocytoma grade IV, *i.e.* glioblastoma, [WHO Brain Tumor Classification (24)].

Example 1

Expression of α2,3-sialyltransferase (α2,3-ST) in glioma

The role of α2,3-ST in carcinogenesis remains unclear to those skilled in the art. Since α2,3-ST mRNA expression is detected in normal human fetal astrocytes, it is possible the α2,3-ST gene is under developmental regulation (Kitagawa, 1994). As gliomas synthesize various extracellular matrix glycoproteins such as fibronectin, collagens, vitronectin and tenascin (Rutka, 1988; Zagzag, 1995), it is also possible that α2,3-linked sialic acids are present on one or more of these proteins and may be involved in tumorigenicity.

A. Detection of α2,3-ST mRNA

To determine whether glioma cells and brain metastases express the α2,3-ST mRNA, northern blot analysis was performed. Thirty μg of total RNA per lane were used for northern analysis. Human α2,3-ST cDNA was cloned by using the reverse-transcriptase polymerase chain reaction (RT-PCR) and poly A+ RNA from U-373 MG cells based on the sequence reported previously (Kitagawa, 1994). A sense primer, 3'-CTGGACTCTAAACTGCCTGC-5' (bp 196-215; SEQ ID NO. 1) and an antisense primer, 5'-CCCAGAGACTTGTTGGC-3' (bp 524-508; SEQ ID NO. 2) were used. 30 pmol each of a sense primer corresponding to SEQ ID NO:1 and an antisense primer corresponding to SEQ ID NO:2 were utilized. The PCR amplification cycle consisted of

denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds and elongation at 71°C for one minute. After 35 cycles, a 329 bp PCR product was subcloned into pT7 Blue T vector (Novagen, Madison, WI) and the sequence of the insert was confirmed by the dideoxy termination method (Sequenase, United State Biochemical, Cleveland, OH). The cDNA coding for human α2,3-ST cDNA was gel purified following Xba I and Bam HI digestion of the vector and used as the template.

A panel of 13 surgical glioma specimens was analyzed in **Figure 4A**: 1 astrocytoma grade II, 1 high-grade oligodendroglioma, 1 mixed glioma, 3 cases of astrocytoma grade III and 7 cases of astrocytoma grade IV, *i.e.* glioblastoma, (WHO Brain Tumor Classification, (Kepes, 1990)). Although the expression appeared variable, it is clear that 12 of 13 gliomas, as well as normal brain, expressed α2,3 ST mRNA. The only negative specimen was the grade II astrocytoma in Fig. 4, lane 2.

To determined if α2,3-ST is expressed in brain metastases, a panel of surgical specimens in **Figure 4B**: 4 adenocarcinomas of lung origin, 3 adenocarcinomas of unknown origin, 1 papillary clear cell tumor of renal origin and one large cell neoplasm of unknown origin were also analyzed using the northern blot. The expression of α2,3-ST in glioma specimens is shown in the upper panel and brain metastases are shown in the lower panel. Seven of the nine samples demonstrate expression of α2,3 ST mRNA. Both negative samples (**Fig. 4B.1**, lanes 2 & 7) were adenocarcinomas of unknown origin.

The expression of α2,3 ST mRNA was also detected in all human glioma and neuroblastoma cell lines examined, and was particularly high in cultured human fetal astrocytes (**Fig. 5A-D**). All established human neural cell lines were maintained using Dulbecco's modified Eagle's medium (DMEM, containing 4.5 g/L glucose) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker BioProducts, Walkersville, MD). Fetal astrocytes were prepared according to a method described previously (Yong, 1992). These data indicate little difference in mRNA expression between glioma specimens and normal brain tissue, included as a control.

B. Detection of α2,3-ST protein

In order to identify the cells expressing glycoproteins bearing α2,3-linked sialic adds, *Maackia amurensis* agglutinin (MAA) lectin staining was performed as described

previously (Wang, 1988). The sections (6 μm thick) were dewaxed, hydrated and soaked in Tris-buffered saline (TBS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) for 1 - 18 hours at 37°C, then incubated in 0.5% blocking reagent (Boehringer Mannheim, Indianapolis, IN) in TBS for 45 mm. After rinsing twice with TBS and once with Buffer 1 (TBS with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5) for 10 mm each, digoxigenin-labeled MAA (Boehringer Mannheim) 10 μg/ml in Buffer 1 was overlaid for 1 h. After washing with TBS (3 X 10 mm), the sections were incubated with anti-digoxigenin Fab-conjugated with alkaline phosphatase (Boehringer Mannheim) at concentration of either 0.75 or 1.5 U/ml TBS for 1 h. After washing three times with TBS, BCIP/NBT solution (Sigma, St. Louis, MO) was overlaid as chromogen for 3-40 mm. The sections were rinsed with deionized water and lightly counterstained with nuclear fast red. Figure 6 illustrates the surfaces of glioblastoma cells (A), extracellular matrices between glioblastoma cells (B) and glioblastoma parenchyma (C) were heavily stained, while vasculatures within the tumors (B, C) remained negative. Positive MAA staining was observed in capillaries of normal cerebral cortex, but not in neurons or glial cells (D).

Figure 6 shows that, while normal adult astrocytes and neurons were not stained with MAA, robust staining of glioblastoma tissue was observed. For example, a glioblastoma specimen (the specimen used in lane 4 of the Northern analysis shown in Fig. 4A) displayed heavy cell-surface staining of pleomorphic tumor cells (**Fig. 6A**) as well as at the invasion front proximal to the surrounding tissue (**Fig. 6C**). In another glioblastoma specimen (lane 10 in Fig. 4B), the matrices of clusters of undifferentiated small cells were stained with MAA, while proliferating endothelial cells derived from glomeruloid neovascularization were not stained (**Fig. 6B**); large vascular lumina in glioblastomas were rarely stained (data not shown).

The predominant MAA-positive cells found in normal adult cerebral cortex and white matter were vascular endothelial cells, suggesting that α2,3-ST activity may play an important role in neovascularization. It should be noted that, because of the inherent limitations in the sensitivity of the detection method used in these studies, it is possible that normal adult astrocytes express α2,3-linked sialoglycoproteins at very low levels. Under the conditions employed in these studies, then, expression of α2,3-linked sialic

acids (as demonstrated by MAA lectin histochemistry) could not be detected in adult human astrocytes; however, robust staining of fetal astrocytes, normal adult brain vascular endothelial cells and primary human glioma specimens was observed. Consistent with such data, α-2,3-ST mRNA expression was observed in human fetal astrocytes, established glioma cell lines, and primary human glioma specimens. α2,3-ST mRNA was detected in whole brain tissue using northern blot analysis. However, lectin histochemical analysis with MAA revealed that only vascular endothelial cells were positively stained. Thus it can be concluded that α2,3-ST mRNA expression in normal adult brain is expressed in vascular endothelial cells and at very low levels, if at all, by normal adult glia.

The differential MAA lectin staining of glioma cell surfaces but not normal adult glia and the heavy MAA staining of glioma-associated extracellular matrices suggests the presence of glioma-associated glycoproteins bearing α2,3-linked sialic acids. α2,3-ST was also found in most of the metastases to the brain. These data indicate that α2,3-ST is found in abundant amounts in malignant brain tumor tissue. It is possible, therefore, that α2,3-ST plays an important role in metastases of tumor cells to the brain. One embodiment of the present invention, then, addresses this possibility by providing a therapeutic treatment comprising administration of reagents that inhibit the function or expression of α2,3-ST in a cell.

Thus, the expression of α2,3-ST in malignant gliomas and other human brain tumor cells provides the possibility that alteration of α2,3-ST expression may alter tumorigenicity of such cells.

Example 2

Development of a glioma cell line expressing α2,6-ST

The α2,6-ST enzyme has been suggested to play an important role in the transformation, metastatic potential and differentiation of colon carcinomas (La Marer, 1992; Le Marer, 1995; Dall'Olio, 1995; Vertino-Bell, 1994; Bresalier, 1990; Sata, 1991). of such cells. In addition, pre-treatment of metastatic colon carcinoma cells with a

sialyltransferase inhibitor results in a significant decrease in pulmonary metastases (Kijima-Suda, 1986). High α2,6-sialylation of N-acetyllactosamine sequences in ras-transformed fibroblasts has been reported to correlate with high invasive potential (La Marer, 1995). Also, increased sialylation of metastatic lymphomas results in reduced adhesion of such cells to extracellular matrix proteins (Dennis, 1982).

Applicants have previously examined the expression of α2,6-ST in a variety of human brain tumors (Kaneko, 1996; Yamamoto, 1995). Applicants did not observe α2,6-ST expression in gliomas or metastases to the brain. These results suggest that a lack of expression of α2,6-ST may correlate with an increased tumorigenicity of gliomas as well as increased potential for metastases of tumor cells to the brain.

Glioma cells have been demonstrated to express extremely low levels of α2,6-ST enzyme in contrast to their normal glial cell counterparts. Based on the hypothesis that a decrease in α2,6-ST may increase the metastatic ability of such cells, one emboidment of the present invention provides a cell line with which that hypothesis may be explored. Such a cell line is a valuable research tool and potentially as part of a therpeutic modality with which a neurological disorder such as a brain tumor may be treated. U373 MG was chosen as a suitable cell line for transfections because it does not express α2,6-ST mRNA or cell-surface linked sialic acid-containing glycoproteins (Kaneko, 1996; Yamamoto, 1995). The methodology with which such a cell line has been developed is demonstrated below.

A. Cell Culture

The human glioma cell line, U373 MG (American Type Culture Collection (ATCC), Rockville, MD) and all transfectants were maintained using Dulbecco's modified Eagle's medium (DMEM, containing 4.5 g/L glucose) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker BioProducts, Walkersville, MD) at 37°C in a humidified 10% CO_2 incubator.

B. Transfections

Human glioma U373 MG cells were transfected with the 1.45 kb rat α2,6-ST cDNA (Weinstein, 1987). For the stable transfections it was inserted into the pcDNA3 expression vector (Invitrogen, San Diego, CA) at the EcoRI site. The orientation of the

insert was confirmed by ApaI restriction digestion. The pcDNA3/α2,6-ST construct was then transfected into U373 MG cells using a cationic liposome system, DOTAP (Boeringer Mannheim, Indianapolis, IN). Putative transfectants were then selected by antibiotic resistance in cell culture medium containing 800 μg/ml G418. After 6 weeks of culture in the presence of G418, the remaining cells were tested for the presence of α2,6-linked sialo-glycoproteins and α2,6-ST mRNA expression.

C. Cell-surface α2,6-linked sialo-glycoproteins are expressed on the cell-surface of the stable transfectant

The transfected cell population was stained for the presence of α2,6-ST protein and α2,6-linked sialoglycoconjugates on the cell surface. Thirty percent of the initial transfectants were positive for α2,6-ST and α2,6-linked sialoglycoconjugates (**Figure 7, C and F**). The transfected cells were also stained with PHA-E lectin, which stains bisecting-type complex oligosaccharides (Cummings, 1982). There was no difference in the PHA-E staining between transfected and non-transfected cells (**Figure 7, A and B**). These data indicate that there is little or no change in the branching pattern of the complex N-linked oligosaccharides after α2,6-ST transfection.

1. Detection by FITC-SNA staining

Expression of cell-surface α2,6-linked sialoglycoconjugates in transfected U373 MG cells was confirmed by staining with FITC-conjugated *Sambucus nigra* agglutanin (FITC-SNA; Vector laboratories, Burlingame, CA) to recognize the terminal Neu5Acα2,6Gal sequence using a modification of previously published methods (Lee, 1989). Preconfluent cells, grown on 12 mm glass coverslips, were fixed with 10% buffered formalin for 20 min at 25^{0}C followed by washing once with PBS. The fixed cells were incubated for 15 min at room temperature with PBS containing 10 μg/ml FITC-SNA (Vector Labs, Burlingame, CA) and 1% BSA. After incubation, excess FITC-SNA was removed by washing the cover slips with PBS three times. The cells were mounted in 70% glycerin. Fluorescence microscopy was performed using a Nikon Model 401 Fluorescence Microscope. The pcDNA transfected cells were used as controls. FITC-PHA-E lectin (Vector Labs) was also used as a control to confirm that the branching of complex-type oligosaccharide structures in the transfectant remained unchanged after

α2,6-ST transfection. This lectin has been reported to stain "bisecting-type", complex oligosaccharides (Cummings, 1982).

2. Detection by anti-α2,6-ST antibody staining

The transfected cells were plated onto 12 mm glass cover slips at 70% confluency, washed with PBS twice, and fixed with 10% buffered formalin for 20 min at room temperature. The fixed cells were washed with PBS once for 3 min and incubated with 1% Nonidet P-40 (Sigma) in PBS for 10 min followed by washing twice with PBS for 3 min, all at room temperature. The cells were then incubated with affinity purified anti-rat α2,6-ST antibody (1:200 dilution) in 10% normal goat serum for 15 min at room temperature. This antibody was generously provided by Dr. Karen Collev (Univ. of Illinois at Chicago). After washing with PBS three times, the cells were incubated with FITC-labeled, anti-rabbit IgG (1:160 dilution; Sigma, St. Louis, MO) in PBS for 1 hr. The cells were washed with PBS three times to remove unbound secondary antibody and were mounted with 70% glycerin. Fluorescence microscopy was performed using a Nikon Model 401 Fluorescence Microscope. The pcDNA3 transfected cells were used as controls.

D. Subcloning of α2,6-ST transfected glioma cells

Sterile bacterial plates were coated aseptically with *Sambucus nigra* agglutanin (SNA)(5 μg/ml), in 50 mM Tris-HCl, pH 9.5, incubated for 2 hrs at 20°C, and washed three times with 10 ml of 0.15 M NaCl. The plates were then incubated with 1 mg/ml BSA in PBS at 4°C overnight to block non-specific binding of the cells. Well-dissociated transfected cells were incubated on the SNA coated plates for 10 min at 20°C. Unbound cells were removed by washing the plate 10 times with PBS. Cells that remained bound to the plate were then allowed to grow by the addition of normal culture medium, and cloning rings (Belco Glass) were used to isolate individual clones.

A total of 36 clones were isolated. Three of these clones were chosen for further analysis. Greater than 95% of the cells in each of these three clones were positive for SNA staining on the cell surface and stained affinity purified anti-α2,6-ST antibody. The intensity of staining, however, differed for each clone. The data for the most intensely stained clone (#35), is shown in **Figure 8A** and **8B**. SNA staining of clone #35 was

predominately on the cell surface but some cytoplasmic staining was also observed (**Figure 8B**). Anti-α2,6-ST staining was localized to a perinuclear intracellular organelle, consistent with Golgi staining (**Figure 8A**). The morphology of this clone is more round and less dendritic than the initial transfectants or controls (**Figure 8, B and D**).

E. Detection of α2,6-ST mRNA in transfectants

Northern analysis was performed to detect the expression of α2,6-ST mRNA in the transfectants. Total RNA was isolated from parental U373 MG cells and transfectants using guanidium isothiocyanate (Chomczynski, 1987) followed by $CsCl_2$ centrifugation (Chirgwin, 1979). 20 μg of total RNA per lane was electrophoresed in a formaldehyde-agarose gel and transferred to Duralon nylon membranes (Stratagene, La Jolla, CA). After UV cross-linking, blots were hybridized with a ^{32}P-radiolabeled rat α2,6-ST cDNA probe synthesized by using a random priming kit (Stratagene, La Jolla, CA) and QuikHyb solution (Stratagene, La Jolla, CA). After washing at 60^{0}C, the blot was exposed to X-OMAT film (Kodak, Rochester, NY) for 16 hours and the film was then developed. Under these stringent conditions, the rat α2,6-ST cDNA probe only weakly cross-hybridized with the human transcript (data not shown).

The expression of rat α2,6-ST mRNA in the transfectants is demonstrated in **Figure 9A**. A 2.1 kb transcript was detected in cells transfected with pcDNA3/α2,6-ST but not in parental cells or pcDNA3 transfected controls. In addition to message expression, α2,6-ST enzyme activity was determined in each of the isolated clones. The relative enzyme activity correlated well with the level of message expression (**Figure 9C**). Clone #35 expressed the highest amount of α2,6-ST mRNA and also had the highest relative enzyme activity. Similarly, clone #24 expressed the least amount of message and had the lowest relative enzyme activity. Consistent with the highest level of message and enzyme activity, clone #35 also stained the most intensely with SNA indicating a high level of α2,6-linked sialoglycoconjugates on the cell surface.

F. Detection of α2,6-ST enzyme activity in transfectants

The α2,6-ST enzyme activity of the transfectants was measured as described by Paulson, et al. (1990) using the sugar nucleotide donor, CMP-(^{14}C)NeuAc (6200 dpm/nmol; NEN/DuPont, Wilmington, DE) and asialo-α1-acidic glycoprotein (50)

kg/reaction mixture; Sigma, St. Louis, MO) as the acceptor. A whole cell extract was used as the enzyme source and the enzyme reactions were run for 30 mm at 37°C and terminated by dilution into 1 ml of ice-cold 5 mM sodium phosphate buffer, pH 6.8. ^{14}C-labeled protein products were immediately separated from unincorporated CMP-(^{14}C)NeuAc by Sephadex G-50 column chromatography and quantitated using a Beckman LS 60005E liquid scintillation spectrometer.

Example 3

Effects of α2,6-ST expression on glioma cell behavior in vitro.

Integrins are a superfamily of transmembrane receptors that participate in cell-cell and cell-matrix interactions (Juliano, 1993; Hynes, 1992; Ruoslahti, 1992; Yamada, 1992). They are heterodimeric glycoproteins in which one of at least 14 α subunits associate with one of at least 8 β subunits to form a functional receptor (Ruoslahti, 1992). Most of the integrins that mediate adhesion to extracellular matrix components contain a common β1 component.

It is understood by those skilled in the art that glycosylation of integrin receptors is important for their function. Decreased sialylation of the β1 integrin subunit has been correlated with decreased adhesiveness and metastatic potential (Kawano, 1993). Furthermore, the ability of α5β1 receptors to form functional heterodimers depends on the presence of N-linked oligosaccharides (Zheng, 1994). Human fibroblasts cultured in the presence of 1-deoxymannojirimycin (DNJ) expressed incompletely glycosylated FN receptors and FN adhesion was greatly reduced (Akiyama, 1989). Adhesion to fibronectin and collagen were reduced more than 50% by treatment of colon carcinoma cells with DNJ (von Lampe, 1993). The α6β1-dependent binding of B16/F10 melanoma cells to laminin was nearly abolished when cells were treated with tunicamycin (Chammas, 1993). Furthermore, enzymatic deglycosylation of the α5β1 integrin receptor abolished its ability to bind to FN (Zheng, 1994).

The interaction of integrins with extracellular matrix components not only provides a structural link with the matrix but also gives rise to biochemical signals. Adhesion to and spreading on extracellular matrix results in the tyrosine phosphorylation

of several focal adhesion proteins, including paxillin, focal adhesion kinase ($pl25^{fak}$; FAK), and tensin (Richardson, 1995; Rosales, 1995; Clark, 1995; Schuppan, 1994). The phosphorylation of FAK is a key component of integrin-mediated adhesion and migration (Richardson, 1995; Rosales, 1995). Activation of both FAK and multiple signaling pathways are required for the appearance of strong cell adhesion, the turnover of focal adhesion sites (Schwartz, 1994; Sankar, 1995). Thus, alteration of integrin function or the signaling mechanisms associated with integrins may alter the adhesion properties of the cell.

DiMilla et al. (1991) and Lauffenberger (1989) have developed theoretical models demonstrating an inverted U-shaped relationship between cellular adhesivity and migration. A reduction in cellular adhesivity brought about by, for example, an alteration in integrin glycosylation, could either enhance or retard cell migration depending upon the initial strength of adhesion between a cell and its substratum. Experimental studies by several groups support this hypothesis: DiMilla et al. (1993) found that an optimal adhesiveness exists for muscle cell migration on collagen; Albeda (1993) and Wu et al. (1994), showed concentration-dependent, inhibitory and enhancing effects of an integrin-binding inhibitor on cell motility (Bresalier, 1990); and Keely et al. (1995), reported that cell motility of mammary cells across collagen-coated filters was increased only in those clones with intermediate levels of adhesion to collagen (see also Akiyama, 1989). Thus, a highly adhesive fibroblast with increased α2,6-sialylated cell-surface glycoconjugates, and reduced adhesivity to fibronectin, would be more invasive (La Marer, 1995). Thus, alteration of a cell's adhesive properties may represent a useful method with which to treat a disease, such as cancer.

A. Invasivity

Invasivity of the U373 MG/α2,6-ST transfected subclones (clones #18, #24 and #35) was examined using a commercial membrane invasion culture system (**Figure 10**; Paulus, 1994; Hendrix, 1989). Biocoat Matrigel Invasion Chambers (Collaborative Research, Bedford, MA) consist of two compartments separated by a filter precoated with Matrigel (contains: laminin, type IV collagen, entactin and heperan sulfate). Cell invasion is measured by counting the number of cells passing to the opposite side of the filter via 8

micron pores. 4 x 10^4 cells were plated into the upper chamber and incubated for 24 hr. 0.5 ml of U373 MG-cell conditioned medium was placed in the lower compartment to facilitate chemoattraction (Hendrix, 1989). Cells that migrated through the Matrigel and through the filter were fixed in 10% formalin and stained with hematoxylin. The membranes were mounted on glass slides and the cells counted (Paulus, 1994).

All data were normalized to pcDNA3 transfected cells. The invasivity of clones #18 and #35 were reduced to less than 20% of control values (**Figure 10**). The invasivity of clone #24 was only reduced to 60% of the control values. These data appeared to correlate with the expression of enzyme activity in these clones (**Figure 9**). Anti-α3 antibody was used to determine if α3β1 integrin was involved in the invasion process. Anti-α3 antibody (Novacastra; clone VM-2) completely abolished invasion of control pcDNA3-transfected cells in this assay.

B. Adhesion

Cell adhesion to defined matrix components was accomplished as previouslv described (Mosmann, 1994). Flat-bottomed, polystyrene, 24-well plates were incubated overnight at 4°C with 40 µg / 250 µl/well of an extracellular matrix substrate. Human fibronectin, human collagen type I, human laminin or human vitronectin (Collaborative Research, Bedford, MA) was used as a substrate. Plates were washed with 500 ml of 1.0 % BSA in PBS twice to remove unbound extracellular matrix proteins and also to block any remaining reactive surfaces. Non-specific cellular binding was determined using wells coated only with 1.0 % BSA. After washing the plates with PBS, 5 x 10^4 cells/well in 250 µl of DMEM were plated and the cells were incubated at 37°C for 10 min or 30 min for attachment to the fibronectin substrate. After washing off non-adherent cells, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5mg/ml) was added to the culture, incubated for 3 hrs, and then 250 µl of acidic isopropanol (0.1N HCl in isopropanol) was added and mixed completely. Optical density (570nm minus 630nm) was measured to evaluate cells attached to the substrate. The cells without the washing procedure were used as 100%.

The U373 MG cells used in these studies express the α3β1 integrin as their only integrin (data not shown). This integrin has been reported to bind type I collagen,

fibronectin, and laminin (Ruoslahti, 1994). The ability of the transfected clones to adhere to these extracellular matrix components was compared to that of untransfected U373 MG cells and pcDNA3 transfected U373 MC cells. Adhesion to a vitronectin substrate was also examined as a non-α3β1-mediated adhesion control. Adhesion was examined after 10 or 30 min incubation of the cells on the coated wells using a colorimetric assay (Kaneko, 1996). At 10 or 30 min incubation, approximately 40-50% of the control cells adhered to fibronectin (**Figures 11C and 11D**) or laminin substrata. On the collagen coated wells (**Figures 11A and 11B**), only 5% adhesion of the control cells was detected at 10 mm. This increased to 10-20% adhesion after 30 min. These data suggested that the kinetics of adhesion to type I collagen were different than that to fibronectin or laminin. A marked reduction in adhesion to both fibronectin and type I collagen substrata was observed with α2,6-ST transfected clones (**Figure 11**) that expressed high amounts of α2,6-ST message and activity (**Figure 9**). The reduction in adhesion to fibronectin was observed at both 10 and 30 min incubation. Consistent with the different binding kinetics to collagen, reduced adhesion of the transfectants to type I collagen was best observed at 30 mm. The reduction in adhesion of the transfectants was correlated with the degree of α2,6-ST expression and inhibition of invasivity (**Figure 10**). Little difference was observed when laminin or vitronectin were used as a substrate (data not shown). These data suggested that α2,6-ST transfection of human glioma cells resulted in differential effects on the adhesion of these cells to different extracellular matrix components.

C. Sialylation

A marked reduction in adhesion to a fibronectin or collagen type I substrate was found in α2,6-ST transfected cells (**Figure 11**), and decreased adhesion was correlated with the degree of α2,6-ST expression (**Figure 9**). The effect of the gene transfection on the level of α3β1 integrin protein was determined in order to rule out the possibility of altered receptor expression as an explanation for the changes in adhesion.

Clone #18 cells and U373 MC/pcDNA3 cells were incubated with methionine-free DMEM and 2 μCi/ml ^{35}S-methionine for 16 hrs, and the cells were harvested. The membrane fraction was isolated, and solubilized with 1% NP-40 in 50 mM Tris-HCl, pH 7.6 containing proteinase inhibitors. 300 μg of solubilized proteins were used for

immunoprecipitation with 20 μl anti-VLA3 monoclonal antibody (Novocastra, clone VM-2) followed by rabbit anti-mouse IgG and Protein A-agarose adsorption. Immunoprecipitated proteins were solubilized with 2% SDS and were loaded on a 6% SDS-polyacrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film (**Figure 12,** lanes 1 and 2). The immunoprecipitated proteins were also transferred to a PVDF membrane after electrophoresis and stained with SNA lectin to detect α2,6-linked sialic acids (**Figure 12,** lanes 3 and 4). Anti-VLA3 antibody recognizes the 140kD α3 integrin subunit and co-immunoprecipitates a 120 kD protein, which is consistent with β1 subunit. α2,6-linked sialylation of α3β1 integrin molecules was detected in the transfectant but not in control cells.

Similar amounts of ^{35}S-labeled α3β1 integrin were immunoprecipitated from both the control and transfected cells (**Figure 12,** lanes 1 and 2). The anti-VLA3 antibody used for this experiment directly recognizes the 140 kD α3 subunit and co-immunoprecipitates an 120 kD protein which is consistent with β1 subunit. These data indicated that there were no large differences in the levels of either the α3 or β1 protein.

The presence of α2,6-linked sialylation on the immunoprecipitated α3β1 integrin receptor in the transfected cells was determined by SNA staining. Abundant SNA staining of both subunits was detected in the transfected cells, while no SNA staining was observed in control cells (**Figure 12,** lanes 3 and 4). These data indicated that α2,6-linked sialylation was present on the α3β1 integrin.

D. Tyrosine phosphorylation

The reduction in adhesion to fibronectin or collagen type I substratum suggested alteration in the ability of α2,6-sialylated integrins to bind. Binding of integrin receptors to their ligands stimulates tyrosine phosphorylation (Richardson, 1995) as well as adhesion to the extracellular matrix. Integrin-mediated protein tyrosine phosphorylation was examined in the transfected clones.

Equal amounts of whole cell lysate (50 μg protein) obtained from the transfected clones and controls were analyzed by SDS-PAGE followed by Western blotting using anti-phosphotyrosine antibody PY20 (Upstate Biotechnology, Lake Placid, NY) as follows. The three subclones and pcDNA3 transfected control cells were incubated in

fibronectin-coated flasks for 10 (**Figure 13A**) or 30 min (**Figure 13B**), and unattached cells were removed by washing three times with cold PBS. The attached cells were then solubilized with 200 µl of lysis buffer. The lysate was centrifuged at 12,000 x g for 5 min to eliminate non-soluble material. An equal amount of protein (50 µg) from each sample was loaded on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane, and the membrane was incubated with 3% non-fat milk at 21°C for 30 min. Anti-phosphotyrosine antibody (Upstate Biotechnology) was then added at 1/1000 dilution and incubated at 21°C for 1 hr. The membrane was then washed three times with PBS containing 0.05% Tween 20, and the antibody-bound proteins were detected using an BCL kit (Amersham).

The qualitative pattern of phosphorylated proteins in each of the clones was identical to those of parental U373 MG or U373 MG/pcDNA3 cells (Figure 13). The quantity of tyrosine phosphorylation in the transfected clones, however, was much less than either of the controls. These results suggested that integrin mediated signaling was also inhibited by α2,6-linked sialylation. One of the phosphorylated proteins has a molecular mass of 125 kD, consistent with focal adhesion kinase ($pl25^{fak}$). Focal adhesion kinase is a key tyrosine kinase involved in integrin mediated signal transduction (Richardson, 1995).

The reduction of adhesion-mediated protein tyrosine phosphorylation may be due to reduced expression of integrin-dependent signaling molecules, such as $pl25^{fak}$, in the transfected clones. To test this hypothesis, the expression of focal adhesion kinase $pl25^{fak}$ mRNA was examined by northern analysis. Northern analysis was performed with a human FAK cDNA probe (**Figure 14, panel A**). Human FAK cDNA was cloned by using the reverse-transcriptase polymerase chain reaction (RT-PCR) and poly A+ RNA from U-373 MG cells. A sense primer, ATGGCAGCTGCTTACCTTGACC (bp 233-254; SEQ ID NO:3) and an antisense primer, TTCATATTTCCACTCCTCTGG (bp 601-571; SEQ ID NO:4) were used (Scirrmacher, 1982; Reboul, 1990). 30 pmol each of a sense primer corresponding to SEQ ID NO:3 and an antisense primer corresponding to SEQ ID NO:4 were utilized. The PCR amplification cycle consisted of denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds and elongation at 71°C for one minute.

After 35 cycles, a 369 bp PCR product (bp 233-601) was cloned into pT7 Blue T vector (Novagen, Madison, WI) and the DNA sequence of the insert was confirmed by the dideoxy termination method. The FAK cDNA was isolated from the gel after Xba I and Bam HI digestion of the vector and used as the template. 20 μg of total RNA per lane was electrophoresed for the analysis. Lane 1, U373 MG cells; lane 2, U373 MG cells transfected with pcDNA3; lane 3, pcDNA3/α2,6-ST transfected clone #18; lane 4, clone #24; lane 5, clone#35. Total RNA staining by ethidium bromide is shown in **Figure 14, panel B**.

All transfected clones showed a marked increase (approx. 10-fold) of $pl25^{fak}$ mRNA expression. $pl25^{fak}$ protein was also increased in these subdones (data not shown) compared to controls. These results suggested that, despite the increased expression of $pl25^{fak}$ in the transfected cells, integrin-mediated stimulation of tyrosine phosphorylation was greatly inhibited.

To characterize the difference between glioma-associated α2,3-ST and α2,6-ST transfected U-373MG clones in adhesion-mediated protein tyrosine phosphorylation, the α2,3-ST and α2,6-ST transfected cells were plated on fibronectin-coated flasks for 30 min, and unattached cells were removed by washing three times with cold PBS. The attached cells were then solubilized with 200 μl of lysis buffer. The lysate was centrifuged at 12,000 x g for 5 min to eliminate non-soluble material. An equal amount of protein (30 μg) from each sample was loaded on an 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane, and the membrane was incubated with 3% non-fat milk at 21°C for 30 min. Anti-phosphotyrosine antibody (PY-20, Upstate Biotechnology) was then added at 1/1000 dilution and incubated at 21°C for 1 hr. The membrane was then washed three times with PBS containing 0.05% Tween 20, and the antibody-bound proteins were detected using an ECL kit (Amersham). Overall protein tyrosine phosphorylation is similar in α2,3-ST and α2,6-ST transfected cells with one exception (**Figure 15**). A phosphorylated protein with a molecular mass of 110 kDa was observed in α2,3-ST cells, but not in α2,6-ST transfected cells. The result suggests that α2,6-ST gene transfection alters adhesion-mediated protein tyrosine phosphorylation.

E. Actin cytoskeletal assembly and focal adhesion formation

The integrin β subunit is primarily involved in integrin-mediated signaling Rosales, 1995). This signaling includes integrin-mediated tyrosine phosphorylation of cytoplasmic proteins, such as focal adhesion kinase $pl25^{fak}$ and reorganization of integrin-cytoskeletal assemblies. The decrease in adhesion mediated phosphorylation or the increased expression of $pl25^{fak}$ may affect integrin and cytoskeletal assemblies including focal adhesion plaques and actin cytoskeletal assembly in the cells.

Human glioblastoma U373 MG cells were transfected with either pcDNA3 (**Figures 16A and 16B**) or pcDNA3/α2,6ST (Clone #18, **Figures 16C and 16D**) were plated on fibronectin-coated cover slips and incubated overnight with DMEM containing 10% FBS. Cells were treated with 1.25 IU/ml cytochalasin D for 1 hr and then fixed with cold methanol for 15 min. After blocking with 10% normal goat serum, the cells were incubated with anti-actin polyclonal antibody (Sigma, St. Louis, MO) at 1:100 dilution for 15 min at room temperature. After washing with PBS three times, the cells were incubated with FITC-labeled, anti-rabbit IgG (1:160 dilution; Sigma) in PBS for 1 hr. The cells then were washed with PBS three times to remove unbound secondary antibody and were mounted with 70% glycerin. Fluorescence microscopy was performed using a Nikon Model 401 Fluorescence Microscope. Phase-contrast photomicrographs are shown in **Figure 16A** and **16C** and actin staining is shown in **Figures 16B** and **16D**.

As previously mentioned, morphological changes were observed in α2,6-ST transfected cells. The cell morphology of α2,6-ST transfected cells is round, and other clones show bipolar, triangular or fan-shaped morphology. As shown in **Figure 17**, α2,6-ST transfected cells grow as mono-layer, while α2,3-ST, vector-transfected control and parental U-373MG cells pile up in culture. The result suggests alterations in cell-cell or cell-extracellular interactions by the α2,6-ST gene transfection in the glioma cells.

To determine whether there is also an effect on cell adhesion and cell spreading, cell spreading was examined in α2,3-ST, α2,6-ST, vector-transfected control and parental U-373MG cells. The most distinct difference was found in α2,6-ST transfected cells after 24 hrs. The α2,6-ST transfected cells showed well-spread round cell morphology, while others showed bipolar or tri-angular morphology (**Figure 18**). The result suggested that

α2,6-ST gene transfection can alter the activation of integrin-cytoskeletal complexes, and change cellular behaviors such as cell adhesion, spreading and invasion.

The observed morphological changes may be and may be due, at least in part, to altered integrin-cytoskeletal assemblies. To examine the possible effects on cytoskeleton, cells were treated with cytochalasin D, to inhibit actin polymerization, and then stained with anti-actin antibody (**Figure 16**). Under these conditions, pcDNA3 transfected control cells maintained their original bipolar or triangular cell morphology and some actin filament structure. On the other hand, the transfected cells had a more rounded or cobblestone morphology. Upon cytochalasin D treatment, the cell body retracted towards the center of the cell with many focal adhesion plaques. No actin fibers were detected and actin staining was only observed at the center of cell body and at focal adhesion plaques.

Example 4

Decreased tumorigenicity of α2,6-ST$^+$ glioma

As demonstrated above, transfection of the α2,6-ST gene into glioma cells caused a marked inhibition of glioma cell invasivity and a significant reduction in adhesivity to the extracellular matrix molecules, fibronectin and collagen. Furthermore, α3β1 integrin was found to contain α2,6-linked sialic acids, and tyrosine phosphorylation of pl25^{fak} was blocked in the transfectants despite increased expression of pl25^{fak} message. These data suggest that glycosyltransferase gene transfections may be a novel way to inhibit or retard glioma invasivity in vivo.

To demonstrate that transfection of a sialyltransferase gene into a glioma cell would result in decreased tumorigenicity, untransfected U373 MG were implanted into a mouse host. Tumor cell growth was compared to that of the α2,6-ST-transfected U373 MG cells.

A. Tumorigenicity of non-transfected vs. transfected glioma cells

1. Loss of tumorigenicity in α2,6-ST transfectants in the nude mouse

Tumorigenicity was evaluated by subcutaneous implantation of α2,6-ST stable transfectants into the hindflank of the nude mouse. Both parental U-373MG cells and vector-transfected controls were confirmed as tumorigenic, while no measurable tumors

were found with α2,6-ST transfected cells (**Figure 19**). Control animals (U-373MG. 10/10) and those injected with pcDNA3 vector-transfected cells (U-373MG/pcDNA3. 10/10) all produced large tumors. α2,6-ST transfected cells produced no measurable tumors in the nude mice (0/10).

2. α2,3-ST Transfection

In vivo tumorigenicity of human U373MG cells stably expressing high levels of transfected α2,3 sialyltransferase was evaluated by subcutaneous implantation into the flanks of nude mice. Three to ten million cells in a 50-100 μl volume were injected into a flank. Although these cells produced no measurable tumors on the flanks, it was noted that after an extended period of time (approximately 4 to 5 months), visible, palpable tumors appeared elsewhere in some of the animals (2/10): one infiltrative spinal tumor and one within the renal capsule. Although no visible tumors could be observed in the other mice, all of the animals demonstrated a significant decline in general health status with time as compared to control mice of similar age. The average body weight of these animals declined to approximately half (12g/26g) over this extended time course. Significant spinal deformation and limb paralysis was also observed in most of the experimental animals. These data are consistent with the *in vitro* experiments demonstrating direct correlation between α2,3 sialyltransferase expression and invasivity. Furthermore, these data demonstrate that alteration of α2,3 sialyltransferase activity in a cancer cell inhibits the tumorigenicity and malignancy of the cell.

B. Altered Sialyltransferase Activity Inhibits Intracranial Tumor Growth

The intracranial tumorigenicity of α2,6-ST transfected U-373MG, α2,3-ST transfected U-373MG, parental U-373MG and pcDNA3 vector-transfected control cells in severe combined immuno-deficient (SCID) mice was determined. 10 μl of a 1.25 x 10^6 glioma cell suspension were injected stereotactically into the right basal ganglia of anesthetized SCID mice (C.B-17 scid/scid, 6 weeks old) and the brains were harvested after six weeks. The brains were mounted on cryostat pedestals and serial 6 μm thick coronal sections were cut through the basal ganglia at 20 μm intervals. The sections were used to determine tumor size by hematoxylin and eosin staining (**Figure 20, A, B** and **C**)

or anti-human EGF-receptor antibody staining (**Figure 20, D, E** and **F**). The maximum cross-sectional area of the tumors was determined by computer-assisted image analysis using the Microcomputer Imaging Device (MCID) software package of Imagimg Research (Brock University, St. Catherines, Ontario, Canada). Ten mice per transfectant were used in each of 4 groups (parental U-373MG glioma cells, three different α2,6-ST transfected U-373MG glioma clones, three different α2,3-ST transfected U-373MG glioma clones, and pcDNA3 vector transfected U-373MG cells as a control) for a total of 80 mice. Difference in tumor size among the animal groups was determined. As shown in **Figure 21**, α2,6-ST transfected U-373MG glioma clones formed virtually no tumors. On the other hand, α2,3-ST transfected U-373MG clones formed smaller tumors than in the vector transfected cells (averaging 10% of pcDNA3 vector transfected U-373MG cells). These results suggest that alterations in cell-extracellular interaction by changing cell-surface sialylation can inhibit tumor formation *in vivo* and that gene therapy with the α2,6-ST gene has potential for the treatment of malignant glioma.

C. Immuno-resistance of α2,6-ST expressing glioma cells

Since malignant gliomas are resistant to T-cell mediated lysis, increased terminal sialylation may be important in their ability to escape immune surveillance.

Example 5

Alteration of Glycosyltransferase Expression in Cancer Cells

The importance of N-linked oligosaccharide branching in tumor metastasis was demonstrated in a series of experiments reported by Dennis and co-workers (14). Specifically, they created a panel of glycosylation mutants was generated in a highly metastatic murine tumor cell line and showed a strong correlation between the increased β1,6-linked branching of complex type oligosaccharides and metastatic potential. A number of more recent studies have also shown an increased expression of highly branched β1,6-GlcNAc linked N-glycans in a variety of tumor models including cells transformed by DNA viruses such as Polyoma and Rous sarcoma, oncogenes such as *H-ras* and *src* and various human breast and colon cancers (3, 15, 16, 22, 30). Furthermore, increased β1,6-GlcNAc linked N-glycans, brought about by GnT-V gene transfection into

premalignant mink lung epithelial cells, resulted in increased tumorigenicity due to an increase in cell motility by alterations in $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins (11). Here, it is demonstrated that N-linked oligosaccharide branching, found on the glioma-associated glycoproteins such as the integrin $\alpha 3\beta 1$, has a significant role in the invasivity and, therefore, tumorigenicity of brain cancer cells.

A. Northern analysis

To address the question as to whether changes in N-glycan branching plays a role in glioma invasivity, an examination of the expression of GnT-III and GnT-V mRNA was undertaken. A 1.24 kb human GnT-V cDNA (SEQ ID NO.: 17) was isolated after Eco RI restriction digestion and used as a cDNA probe for northern analysis. A 1.8 kb human GnT-III cDNA (SEQ ID NO.: 15) was used as a probe after Eco RI and Xba I restriction digestion.

Surgical specimens were immediately frozen in liquid nitrogen upon resection. Total RNA was isolated from clinical glioma specimens and cultured brain tumor cells using guanidium isothiocyanate followed by $CsCl_2$ centrifugation using standard techniques. 30 µg of total RNA per primary brain tumor and 20 µg of total RNA per tumor cell line per lane were electrophoresed in an agarose-formaldehyde gel and transferred to Duralon nylon membranes (Stratagene, La Jolla, CA). After UV cross-linking, the blots were hybridized with a ^{32}P-radiolabeled cDNA probe synthesized by using a random priming kit (Stratagene, La Jolla, CA) and ExpressHyb solution (Clontech, Palo Alto, CA). The blots were then exposed to X-OMAT film (Kodak, Rochester, NY) and the films were developed appropriately.

In normal adult human brain, robust GnT-III mRNA expression was observed whereas GnT-V mRNA expression was very low by comparison. In the malignant gliomas examined, both GnT-III and GnT-V mRNAs were variably expressed. Most of the clinical specimens used in this study were high-grade gliomas. Patients with these tumors have the shortest survival (6-12 months upon diagnosis). In glioma cell lines, GnT-III mRNA levels were uniformly high, while GnT-V mRNA levels were quite variably expressed (**Figures 22** and **23**).

B. Lectin histochemistry with *Phaseolus vulgaris* leukoagglutinating lectin (L-PHA)

Lectin staining with L-PHA, which recognizes β1,6-GlcNAc containing oligosaccharides, was performed on tissue sections and cultured cells to determine where these structures are expressed. β1,6-GlcNAc expression in primary glioma specimens was examined using *Phaseolus vulgaris* leukoagglutinating lectin (26). To study tissue sections, paraffin embedded sections (6 μm thick) of formalin-fixed specimens, derived from 1 mixed glioma case, 2 cases of astrocytoma grade III and 2 cases of glioblastoma (astrocytoma grade IV), were processed at room temperature unless otherwise mentioned. The sections were dewaxed and hydrated, then soaked in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) at 37 °C for 1 h or 13 h (according to our preliminary studies with other lectins) to unmask lectin binding sites.

The sections were then rinsed with TBS for 10 min and incubated in 0.5% blocking reagent (Boehringer Mannheim, Indianapolis, IN) in TBS for 45-60 min. After rinsing twice with TBS and once with Buffer 1 (TBS with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5) for 10 min each, 10 μg/ml digoxigenin-labeled L-PHA (Boehringer Mannheim) in Buffer 1 with or without 0.05% Tween 20 and 0.05% Triton X-100 was overlaid for 1 h. Rinsing with TBS (3 X 10 min) was followed by incubation with anti-digoxigenin Fab fragments conjugated with 0.75 U/ml alkaline phosphatase (Boehringer Mannheim) in TBS containing 0.05% Tween 20 and 0.05% Triton X-100 for 1 h. After rinsing (TBS , 3 X 10 min), BCIP/NBT solution (Sigma, St. Louis, MO) was overlaid as chromogen in darkness up to 50 min and rinsed with 10 mM Tris-HCl with 1 mM EDTA. The sections were lightly counterstained with nuclear fast red, and fixed with 10% buffered formalin to lessen fading of reaction product during dehydration and clearing. To confirm the specificity of lectin binding, each staining was performed simultaneously with labeled L-PHA that was preincubated in the presence of 9 μM bovine thyroglobulin (Sigma) for 90-120 min prior to lectin incubation as a negative control.

To detect β1,6-branched N-glycans in cultured cells, the cells were rinsed twice with PBS and lysed in hot cell lysis solution containing 1% SDS, 10 mM Tris-HCl pH 7.4. To detect β1,6-GlcNAc N-glycans, 30 μg of cell lysates were loaded on an 8% SDS-

polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane and the membrane was blocked with 5% BSA in PBS. It was then incubated with 0.1 μg/ml horseradish peroxidase-conjugated L-PHA (EY Laboratory, CA) in TBS containing 2% BSA and 0.1 % Tween 20 for 1 h at room temperature. Next, the membrane was washed with TBS containing 2% BSA and 0.1 % Tween 20 for 10 min, followed by washing twice with 0.1 % Tween 20 in TBS. The blot was then developed with the ECL Chemiluminescence detection system (Amersham, UK). Protein concentrations were determined using the BCA reagent (Pierce). Expression of β1,6-branched N-glycans was observed in both glioma cells and neovascular endothelial cells.

L-PHA staining was found in malignant glioma cells, neovascular endothelial cells, and extracellular matrices surrounding the tumor cells, but not in normal cells (**Figure 24**). An L-PHA lectin blot revealed that most glioma cells express a major L-PHA-reactive glycoprotein with a molecular weight of 140 kDa, while protein extracts from neuroblastoma cells or normal brain showed different patterns of L-PHA staining and the 140kDa glycoprotein was rarely found **(Figure 25)**. The expression of the L-PHA-reactive glycoprotein was high in SW1088 and U-87MG glioma cell lines, which show high levels of GnT-V expression, while a small amount of L-PHA reactivity was found in U-118MG glioma cells despite its high GnT-V mRNA expression. Furthermore, neuroblastoma cell lines with high GnT-V mRNA expression (LAN-5) show little or no 140kDa staining. These results suggest that the levels of β1,6-GlcNAc-bearing N-glycans in gliomas are controlled by mechanisms that regulate both GnT-V expression and the availability of its protein substrates. Data obtained from immunoprecipitaion studies using anti α3-integrin antibodies showed that the major glycoprotein recognized by L-PHA in gliomas is α3β1 integrin (data not shown), the most predominant integrin found in clinical gliomas specimens (Paulus, et al. 1993 and 1994) and the U-373MG glioma cell line used in these studies (Yamamoto, et al. 1997). A very recent study has identified that α3 integrin mRNA expression appears to be quantitatively correlated with the grade of malignancy of gliomas and medulloblastomas (Kishima, et al. 1999).

Thus, β1,6-GlcNAc-bearing oligosaccharides were found on the α3β1 integrin and appeared to be associated specifically with gliomas and not normal astrocytes.

Furthermore, aberrant up-regulation of GnT-V expression, as opposed to decreased GnT-III expression, appears to be responsible for their expression. Since GnT-III and GnT-V are the two enzymes that regulate the type of branching structures found within N-linked oligosaccharides, and compete for the same substrates, the results suggest that a mechanism exists to shift the integrin oligosaccharides from bisecting β1,4-GlcNAc to highly-branched β1,6-GlcNAc during the transformation of glia into gliomas or non-invading glioma cells into invasive ones (see Example 9E, below).

C. GnTV and GnTIII Regulate Invasion by Brain Cancer Cells

To study the biological effects of aberrant β1,6-GlcNAc-bearing N-glycan in gliomas, the GnT-V gene was stably transfected into U-373MG glioma cells which express very low levels of this mRNA. The 2.4 kb human GnT-V cDNA (full coding sequence) was inserted into the pcDNA3 expression vector (Invitrogen, San Diego, CA) at the Kpn I and Xba I sites, and the orientation of the insert was confirmed by Hind III restriction digestion. The pcDNA3/GnT-V was then transfected into U-373MG cells using the cationic liposome system, DOTAP, (Boehringer Mannheim, Indianapolis, IN) according to the methods described previously (Yamamoto, et al. 1997). After 3 weeks of culture in selection medium containing 800 μg/ml of G418, transfected cells were subcloned with cloning rings to isolate individual clones. Individual clones were further cultured for 4 weeks in the selection medium and then analyzed for the gene expression by Northern analyses and L-PHA lectin blotting to identify successful GnT-V transfectants (**Figure 26**). Stable transfection of *GnT-III* gene into the same U-373 MG was reported previously (Rebbaa, et al. 1997).

To characterize the morphological change of GnT-V and GnT-III transfectants, immunofluorescence microscopy was performed using monoclonal anti-human vinculin antibody (Sigma, clone hVIN-1) and monoclonal anti-VLA3 antibody (Chemicon, clone M-KD102) (**Figure 27 and Figure 28**). Anti-vinculin antibody was used to visualize focal adhesion sites and anti-VLA3 antibody was used to visualized α3β1 integrin in the transfectants. Cells were plated on fibronectin-coated (10 μg/ml) coverslips and incubated in DMEM supplemented with 10% FBS for 16 h. Cells were gently washed twice with PBS, then fixed with 4% formalin in PBS for 30 min followed by washing

with PBS for 3 min. Cells were treated with 1% NP-40 in PBS for 10 min followed by washing with PBS three times. After blocking with 10% normal goat serum for 15 min at room temperature, cells were incubated with monoclonal anti-human vinculin antibody (1: 400 dilution) or monoclonal anti-$\alpha 3\beta 1$ integrin antibody (1: 200 dilution) in PBS for 30 min at room temperature. They were then washed three times with PBS (5 min each), and then incubated with FITC-labeled goat anti-mouse immunoglobulin antibody (1: 160 dilution, Sigma) for 30 min at room temperature. The cells were washed with PBS five times to remove unbound secondary antibody and were mounted with Vectashield (Vector). Fluorescence microscopy was performed using a Nikon Model 401 Fluorescence Microscope.

Invasivity of the GnT-V transfected subclones was examined using a commercial membrane invasion culture system (Paulus, 1994; Hendrix, 1989) (**Figure 29**). Biocoat Matrigel Invasion Chambers (Collaborative Research, Bedford, MA) consist of two compartments separated by a filter precoated with Matrigel (contains: laminin, type IV collagen, entactin and heperan sulfate). Cell invasion, which is the result of cell adhesion to the extracellular matrix, degradation of the matrix proteins and cell migration to the other side of the filter, is measured by counting the number of cells passing to the opposite side of the filter via 8 micron pores. 4×10^4 cells were plated into the upper compartment and incubated for 24 h. 0.5 ml of U-373 MG cell conditioned medium was placed in the lower compartment to facilitate chemoattraction (Hendrix, 1989). Cells that migrated through the Matrigel and through the filter were fixed in 10% formalin and stained with hematoxylin. The membranes were mounted on glass slides and the cells counted (Paulus, 1994). Parental U-373MG and pcDNA3 vector transfected cells were used as controls.

Directed cell migration studies were also performed. Directed cell migration on a solid-phase gradient of a fibronectin substrate (haptotaxis) was measured using Transwell (Costar, Cambridge, MA) which consist of two compartments separated by 6.5 mm inserts with 8 μm pore polycarbonate filters in 24-well culture plates. To establish a solid-phase gradient, only the underside of the filter was coated with 10 μg/ml human plasma fibronectin (Life Technologies, Grand Island, NY) in sodium bicarbonate buffer, pH 9.7

overnight at 4 °C. It was then blocked with 1% BSA (fatty acid free; Sigma) in PBS for 45 min at RT and rinsed three times with PBS. **(Figure 30).**

For the migration assays, GnT-V transfected, GnT-III transfected U-373MG and control cells were gently treated with X 0.5 trypsin-EDTA (Life Technnologies) in PBS for ~5 min at 37 °C, then neutralized with DMEM containing 0.2% BSA. After washing with 0.2% BSA-DMEM, cells were resuspended in protein free DMEM and were plated 10,000 cells/100 µl/insert. The inserts were moved onto the lower wells which contained protein free DMEM (0.5 ml) and were incubated for 6 h at 37 °C in CO_2 incubator. For inhibition of cell migration by lectins, L-PHA or E-PHA (Vector Laboratory) at the final concentration of 2 µg/ml or 10 µg/ml was added to both upper and lower compartments. Monoclonal anti-α3 integrin antibody (Chemicon, clone P1B5) was also used to inhibit α3β1 integrin-mediated cell migration. After thorough absorption of DMEM with cotton swabs, the porous filter was dried with air blow and cut from the plastic supports. Cells on both sides of the filter were fixed and stained with DiffuQuick (Baxter, Chicago, IL). The filters were then mounted with Parmount (Fisher Scientific, Chicago, IL) on glass slides with 12 mm cover slips. Under the microscope, cells on both the topside (i.e. non-migrated) and underside (i.e. migrated) of the filters were counted in 8 consecutive fields along one filter diameter (~10% of the entire surface was observed). % migration (migrated cell count / total cell count) was determined based on triplicate experiments.

As predicted from the results shown above, GnT-V transfectants were more invasive than controls. These transfectants showed the distinct fan-shaped morphologies indicative of directional cell migration with a distinct leading edge. It has been reported that small numbers of glycoproteins, particularly those involved in adhesion, can be found at the leading lammellipodia in locomoting cells (Kucik, 1991). In the results reported here, α3β1 integrin was found to be localized on the leading lammellipodia of the GnT-V transfected cells and focal adhesion sites radiated toward leading lammellipodia, while parental cells or vector-transfected controls did not show characteristics of migrating cells. Thus, it would be beneficial to block or inhibit GnTV expression in order to treat glioma.

In contrast, GnT-III stable transfectants displayed decreased cell migration under the conditions described above (data not shown). Although the data were not presented, this is likely due to an increase in their adhesion to the fibronectin substratum used in these studies. Thus, GnTIII may be introduced into glioma cells to inhibit tumorigencity.

F. Further Observations

Thus, when all of the data presented here are taken in whole, it suggests that, (a) cell-surface expressed glycoproteins bearing "brain-type" bisecting β1,4-GlcNAc structures, the products of GnT-III, may be directly involved in cell adhesion and migration and (b) the shift of N-glycans from bisecting to highly-branched β1,6-GlcNAc structures on the glycoproteins may function to reduce adhesivity and increase migration, thus increasing cell invasivity. The increased invasivity found in GnT-V transfected clones may be due to altered interaction between α3β1 integrin and the laminin substrate of that integrin, which is a matrix component in the invasion assays. The interaction between α3β1 integrin and appropriate substrata, such as laminin and fibronectin, may be dependent on the N-glycans.

To test this hypothesis, as shown above, *in vitro* migration assays were performed using E-PHA and L-PHA lectins which bind to bisecting β1,4-GlcNAc or highly-branched β1,6-GlcNAc-bearing N-glycans on glycoproteins, respectively. We have previously reported that E-PHA lectin had a marked effect on adhesion in U-373MG cells (Rebbaa, 1996). On the other hand, L-PHA lectin showed no effect on either cell adhesion (Rebbaa, 1996) or cytotoxicity in glioma cells; cytotoxicity was seen in highly metastatic tumor cell lines (Demetriou, 1995; Dennis, 1982). In solid phase cell migration (haptotaxis) studies, E-PHA lectin completely abolished glioma cell migration on fibronectin substrata regardless of the levels of β1,6-GlcNAc expression in both U-373MG transfectants and other glioma cell lines, while migration of glioma cells with high levels of β1,6-GlcNAc N-glycans was weakly inhibited by L-PHA. Furthermore, the inhibitory effect by E-PHA was comparable to that of anti-α3 integrin monoclonal antibody. These data suggest that β1,4-GlcNAc N-glycans play a direct role in α3β1 integrin-mediated cell adhesion, whereas in gliomas, the observed shift to more highly-branched β1,6-GlcNAc N-glycan reduces cell adhesivity and increases invasivity by

replacing functional β1,4-GlcNAc-bearing N-glycans on the adhesion molecules. The binding of E-PHA to β1,4-GlcNAc-bearing N-glycans interferes with cell adhesion (Rebbaa, 1996), thus inhibiting cell migration as shown in this study. On the other hand, L-PHA binding to β1,6-GlcNAc-bearing N-glycans does not interfere with integrin function, and therefore has little effect on cell migration. The results presented here are consistent with previous studies that: (A) N-glycans on α5β1 integrins are required for the functional heterodimerization of integrin α and β subunits (10) and (B) a shift of integrin N-glycans to highly-branched β1,6-GlcNAc leads to decreased cell adhesion resulting in an increase in cell motility by altering the function of α5β1 and αvβ3 integrins (Demetriou, 1995).

In conclusion, the data presented here show that a shift in the expression of normal "brain type" bisecting β1,4-GlcNAc to highly-branched β1,6-GlcNAc N-glycans plays an important role in modulating the function of cell-surface glycoproteins involved in glioma invasivity. A recent study suggests that the knock-out of *GnT-V* gene results in the suppression of both breast tumor formation and lung metastases in the null mouse (Granovsky, 1998). Likewise, the expression of bisecting β1,4-GlcNAc N-glycans by GnT-III gene transfection has been reported to suppress lung metastasis of B16 melanoma (Yoshimura, 1995). The data provided herein suggests that reversion from aberrant β1,6-GlcNAc expressing N-glycans to normal β1,4-GlcNAc-bearing N-glycans can retard glioma invasivity *in vivo*.

Taken together, the data provided by Examples 1-5 above provide significant evidence that modification of cell surface glycosylation provides an effective therapy for brain cancer.

Example 6

Vectors for Delivery of Nucleic Acids Encoding A Glyco-Enzyme

It has been determined that the Coxsackie adenovirus receptor (CAR) protein (36) and RDG-binding protein (such as αv integrins)(Kucik, 1991) are co-receptors for adenovirus infection into cells, and it is well established that both glioma cells and neovascular endothelial cells express αv integrins. Glioma cells are highly sensitive to

infection by human adenovirus serotype 5 (Ad5), which is widely used in human gene therapy. Adenovirus-based systems are capable of producing higher levels of virus titer and gene expression than other gene delivery systems such as herpes simplex virus (HSV) or liposome-based systems. Both replication competent and replication-deficient adenovirus can infect non-dividing and dividing cells and have been used for gene therapy clinical trials. Modified replication-competent viruses, such as Onyx-015, have a cytopathic effect on p53 mutated cancer cells due to a unique molecular mechanism, whereas replication-deficient viruses have been used to deliver genes of therapeutic potential. Adenovirus-based systems do have the potential risk of occasional viral integration, thus causing virus-mediated oncogenic transformation or inducing inflammatory responses such as virus-related demyelination in the brain (Wasylyk, 1990). To minimize such risk factors, we have chosen a replication deficient E1 deleted Ad5 virus carrying the α2,6-ST gene (Adα2,6ST59) as the delivery system.

As shown below, infection of U-373MG cells with a replication-deficient adenovirus carrying the α2,6-ST gene resulted in dose and time dependent: (1) expression of cell-surface α2,6-linked sialic acids, (2) alterations in focal adhesions, and (3) inhibition of invasion *in vitro*. The data suggests that alteration of glyco-enzyme activity in a cancer cell by delivering a glycoenzyme-encoding nucleic acid is a useful method for treating cancer.

A. Ad Vector

Construction of an adenoviral vector encoding a glysosyltransferase gene (α2,6-ST) the adeno/α2,6-ST vector is shown in **Figure 31**. Rat Pα2,6-ST cDNA was excised from the plasmid by Eco RI restriction digestion and ligated into the shuttle vector pCMV-G (**Figure 31A**) at the Hind III site after blunt-ending with DNA polymerase (Klenow fragment). Orientation of the α2,6-ST insert was confirmed by Apa I and Xho I restriction digestion. 25-40 μg of the pCMV-G/α2,6-ST plasmid was then digested with Cla I and Xba I restriction enzymes and the 3.3kb fragment, which contains the viral packaging sequence and α2,6-ST cDNA (SEQ ID NO.: 19 (GenBank Accession No. L29554); coding sequence for α2,6-ST protein found at nucleotides 226-1143), was purified. 50 μg of wild type adenovirus DNA (Ad5 (309/356) was also digested with

XbaI to remove the 1.4kb 5' fragment containing the E1A sequence (**Figure 31B**) and the 35kb adenovirus DNA fragment was purified. 4 μg of the 3.3kb DNA from the shuttle vector and 41 μg of the 35kb DNA fragment were then ligated with T4 DNA ligase at 12°C for 24 hrs with the addition of the ligase every 3 hrs to generate Adα2,6ST59 (**Figure 31C**).

The ligation mixture was then transfected into 293 cells with a cationic liposome system, DOTAP (Boehringer Mannheim, Indianapolis). Typically, 45μg of the Adα2,6-ST59 plasmid DNA was dissolved in 450 μl of Hepes buffer (pH 7.4) and was gently mixed with 900 μl of DOTAP solution (270 μl of DOTAP and 630 μl of Hepes buffer) for 15 min at room temperature. The mixture was then diluted with 20 ml of serum-free DMEM and added to 293 cells in a 150 mm tissue culture dish. After incubation in a 10% CO_2 incubator for 6 hrs, the transfection medium was replaced by normal growth medium (DMEM containing 10% FBS). The transfected 293 cells were maintained until a cytopathic effect (CPE) was observed (typically 7-10 days). The transfected cells were then harvested and the crude virus mixture was extracted from the cells by repeated freeze-thawing. The crude virus extract was again applied to new 293 cells to amplify the virus titer and incubated for 48 hrs until a CPE was observed. The 293 infected cells were harvested and the crude virus stock was stored in a 15% glycerol solution at -20°C. 200 μl of 10^5-10^8-fold dilution virus stock was applied to a new batch of 293 cells (70-80% confluent) in a 60mm culture dish and incubated for 1 hr. The culture dish was then aspirated and cells overlaid with 0.75% bacto-agar containing culture medium. After 8-10 days of incubation, each plaque was punched out by pipets and the virus was extracted from each plaque. Each virus clone was then re-infected into 293 fresh cells and incubated until a CPE was observed. This expansion step was repeated as needed to obtain sufficient quantities of each clone.

To determine whether the Adeno/α2,6-ST virus was successfully generated, virus DNA was isolated and used for PCR analysis using the appropriate restriction digestion protocol. Following confirmation that the vector preparation contained Adeno/α2,6-ST virus, high-titer viral stock was then added to a plate containing 293 cells at 70-80% confluence in infection media (minimal essential medium and 2% fetal bovine serum).

After a 90-minute incubation period, complete media is added to each plate and the cells incubated for 24 to 36 hours until a cytopathic effect is observed. The cells were then harvested and resuspended in five ml of supernatant. To release the virus, the cells were alternately frozen and thawed five times to develop a crude viral lysate. The crude viral lysate was then overlayed on a cesium chloride density gradient and ultracentrifugation performed at 25,000 rpm for 24 hours. The adenovirus was then collected from the gradient with a 21-guage needle and dialyzed three times for four hours each time into 10 mmol/L Tris, pH 7.4, 1 mmol $MgCl_2$, and 10% (vol/vol) glycerol. The virus was then recovered, and stored at -70°C.

B. Adenovirus-Mediated α2,6-ST Gene Expression

(1) Expression of cell-surface α2,6-linked sialic acids in U-373MG glioma cells infected by Adα2,6ST59. U-373MG glioma cells were exposed to an appropriate concentration of Adα2,6ST59 virus for 1 hour at 37°C, and the virus containing media was removed by aspiration. The cells were then washed twice with PBS and returned to normal cell culture media. Expression of α2,6-ST mRNA in U-373MG cells infected with Adα2,6ST59 was confirmed by Northern analysis. Figure 24 shows a dose-response curve over a multiplicity of infection (MOI) range of 0.02 pfu/cell to 200 pfu/cell. The 2.1 kb transcript is detectable at 0.2 pfu/cell and is markedly expressed at 2.0 pfu/cell. A lectin-stained Western blot of the same Adα2,6ST59 infected cells shows detectable SNA lectin staining at 2.0 pfu/cell, indicating the presence of increasing amounts of α2,6-linked glycoproteins (**Figure 32**). **Figure 33** shows the dose-dependent expression of α2,6-ST mRNA in U-373MG cells. **Figure 34** shows time-dependent expression of α2,6-ST mRNA following infection with Adα2,6ST59 at 10 pfu/cell. α2,6-ST mRNA expression is detectable as early as 6 hours post-infection, while a robust expression is seen at day 1 and lasts at least eight days. Based on SNA lectin blotting of the same samples, expression of α2,6-linked sialic acids begins at day 1 and lasts at least 8 days (**Figure 35**).

(2) Alterations in focal adhesions in U-373MG glioma cells by Adα2,6ST59 infection. It has been demonstrated herein that stable transfection of the α2,6-ST gene

resulted in morphological changes, altered adhesion-mediated protein tyrosine-phosphorylation and increased expression of p125fak mRNA. It has been determined that Adα2,6ST59 virus infection results in both morphological changes (**Figure 36**) and increased expression of p125fak mRNA (**Figure 37**) in the same glioma cells. The levels of p125fak mRNA expression are positively correlated with the levels of α2,6-ST mRNA expression.

(3) Inhibition of U-373MG glioma cell invasion *in vitro* by Adα2,6ST59 infection. Invasion of U-373MG cells was inhibited by infection with increasing amounts of Adα2,6ST59 (**Figure 38**). At 10 pfu/cell, *in vitro* invasion of U-373MG cells was decreased by 24%, while at 40 pfu/cell it was only about 8% of the control, non-virus infected U-373MG cells. Infection of the same U-373MG cells with another virus, AdCMVβ2, which contains but does not express the β-galactosidase gene, has little effect on cell invasion, except at the high dose of 40 pfu/cell, wherein invasion is about 65% of the non-virus infected cells.

The biological effects of Adα2,6ST59 infection on U-373MG glioma cells are consistent with our previous observations and suggest that if the α2,6-ST gene can be effectively delivered to glioma cells by Adα2,6ST59, the resulting alterations in cell-surface glycosylation can lead to both inhibition of invasivity and loss of tumorigenicity *in vivo*.

Example 8

Treatment of established tumors in a mammal using nucliec acid encoding a glyco-enzume

The present invention may be utilized to treat a neurological disorder, exemplified herein using a rat brain tumor model. U373 MG cells are counted and resuspended in an appropriate physiologically acceptable buffer such as Hank's balanced salt solution (HBSS). The rat is anesthetized by administration of a composition comprising ketamine and placed into a stereotaxic frame. An incision is made in the scalp, and a burr hole of sufficient diameter is made using a dental drill. Using a 10 μl syringe fitted with a 26 gauge needle and connected to the manipulating arm of the stereotactic frame, U373 MG

cells (5×10^5 to 10^6 cells in 7 µl HBSS) are injected in 0.2 µl increments over 5 minutes into the brain tissue at a depth of 4.5 mm from the dura. The needle is left in place for three minutes and then withdrawn over another three minutes. The burr hole is closed with bone wax and the scalp wound closed with clips. Tumors are then allowed to form within the brain until treatment as described below.

Stereotactic injection is utilized to administer a recombinant adenoviral vector ("Ad2,6") comprising a nucleic acid encoding α2,6-ST under the transcriptional control of the human CMV immediate-early enhancer/promoter into an established U373 MG tumor in a rat brain. Stereotactic injection of a composition comprising the recombinant adenoviral vector is performed. "Treated" animals are injected with a composition comprising 1.2×10^9 Ad-2,6 particles, and "untreated" animals are injected with a composition comprising 1.2×10^9 non-recombinant Ad viral particles (i.e., that from which Ad-2,6 was derived). The viral particles are suspended in 6 µl of 10 mM Tris-HCl, pH 7.4, 10% glycerol, and 1 mM $MgCl_2$ and injected at multiple sites within the tumor bed. Beginning at 5.5 mm below the dural surface, one µl is injected; the needle is then raised 0.5 mm and one µl is injected. A total of six injections are made. Virus injection takes place over five minutes and the needle is removed over five minutes. Carbon particles are placed over the shaft of the injection needle to mark the injection site and the wound is closed with clips. Following administration of the adenoviral particles to the tumors, the effectiveness of the treatment is determined by measurement of tumor growth in treated vs. untreated animals. It is demonstrated that treated animals exhibit less tumor growth than the untreated animals, thus indicating that expression of 2,6-ST in a brain tumor results in a decreased ability of a brain tumor to thrive.

Example 9

Method for prevention of brain tumors following surgical resection of tumor

The reagents and methodologies provided by the present invention are useful for prevention of neurological disorders, exemplfied herein by prevention of tumor recurrence following surgical resection of a brain tumor. Following administration of general anesthesia, a craniotomy is performed on a patient having a glioblastoma brain

tumor. The exact location of the brain tumor is determined prior to surgery using an MRI. As much as possible of the brain tumor is then surgically removed. Following removal of the tumor, a pharmaceutical composition comprising the Ad2,6 viral vector suspended in a liposomal formulation (DOTAP in saline) is applied to the area from which the tumor was removed. The amount of viral particle to be applied may vary but every attempt is made to apply the greatest number of viral particles in as small a volume as possible. The titer of the pharmaceutical composition is optimally 10^{6}-10^{12} viral particles/ml. The effectiveness of the treatment is measured by MRI scanning of the patient's brain at sufficiently timed intervals (optimally, once per week for one year) to determine that tumor cells have not begun to proliferate.

While a preferred form of the invention has been shown in the drawings and described, since variations in the preferred form will be apparent to those skilled in the art, the invention should not be construed as limited to the specific form shown and described, but instead is as set forth in the claims.

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